



IMPERIAL INSTITUTE  
OF  
AGRICULTURAL RESEARCH, PUSA.







THE  
BIOCHEMICAL  
JOURNAL

**CAMBRIDGE UNIVERSITY PRESS**  
**LONDON: FETTER LANE, E.C. 4**



**H. K. LEWIS AND CO., LTD., 136, GOWER STREET, LONDON, W.C. 1**  
**WHELDON AND WESLEY, LTD., 2-4, ARTHUR STREET, NEW OXFORD**  
**STREET, LONDON, W.C. 2**

**CHICAGO: UNIVERSITY OF CHICAGO PRESS**  
**(AGENT FOR THE UNITED STATES)**

**BOMBAY, CALCUTTA AND MADRAS: MACMILLAN AND CO., LTD.**  
**TOKYO: THE MARUZEN-KABUSHIKI-KAISHA**

*All rights reserved*

# THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

HAROLD WARD DUDLEY

AND

ARTHUR HARDEN

EDITORIAL COMMITTEE

PROF. G. BARGER

SIR F. G. HOPKINS

PROF. V. H. BLACKMAN

SIR F. KEEBLE

MR J. A. GARDNER

PROF. W. RAMSDEN

SIR E. J. RUSSELL

VOLUME XXI 1927

CAMBRIDGE

AT THE UNIVERSITY PRESS

1927

**PRINTED IN GREAT BRITAIN**

# CONTENTS

## No. 1

PAGE

I. A Study of the Variations in the Chemical Composition of Normal Human Colostrum and Early Milk. By M. F. LOWENFELD, S. T. WIDDOWS, M. BOND and E. J. TAYLOR. (With two figures) . . . . .	1
II. A Note on the Significance of Gelatin for Bacterial Growth. By B. S. PLATT . . . . .	16
III. Peroxide Formation by Pneumococcus and its Relation to Bacterial Oxidation-Reduction Reactions. By B. S. PLATT. (With three figures) . . . . .	19
IV. A Note on Plant Oxidation: the Nature and Reactions of the Substance "Tyrosin." By B. S. PLATT and A. WORMALL . . . . .	26
V. The Micro-estimation of Chlorine in whole Blood, Serum or Corpuseles. By F. H. SMIRK. (With one figure) . . . . .	31
VI. The Micro-estimation of Iron in Blood. By F. H. SMIRK . . . . .	36
VII. Studies in Carbohydrate Metabolism. II. Influence of Methylglyoxal and other possible Intermediaries upon Insulin Hypoglycaemia. By W. O. KERMAK, C. G. LAMBIE and R. H. SLATER . . . . .	40
VIII. The Heat-denaturation of Proteins. Part IV. The Free Basic and Acidic Groups of Fresh and Denatured Haemoglobin. By P. S. LEWIS. (With two figures) . . . . .	46
IX. The Fate of Desoxy-glucose in the Rabbit. By L. B. WINTER . . . . .	54
X. Studies on the Kinetics of Haemolytic Systems. II. The Series of Ryvosh. By E. PONDER . . . . .	56
XI. The Biochemistry of the Aqueous Humour. By S. DUKE-ELDER . . . . .	66
XII. The Absorption Spectrum of Cholesterol and its Biological Significance with Reference to Vitamin D. Part I. Preliminary Observations. By I. M. HEILBRON, E. D. KAMM and R. A. MORTON. (With six figures) . . . . .	78
XIII. The Feeding of Xanthophyll to Rats on a Diet Deficient in Vitamin A. By S. G. WILLIMOTT and T. MOORE . . . . .	86
XIV. The Tyrosinase-tyrosine Reaction. VI. Production from Tyrosine of 5 : 6-Dihydroxyindole and 5 : 6-Dihydroxyindole-2-carboxylic Acid - the Precursors of Melanin. By H. S. RAPER . . . . .	89
XV. The Constitution and Synthesis of Spermidine, a Newly Discovered Base Isolated from Animal Tissues. By H. W. DUDLEY, O. ROSENHEIM and W. W. STARLING . . . . .	97
XVI. Studies on the Growth of Yeast. III. A Further Study on the Influence of Volume of Medium Employed. By G. L. PESKETT. (With two figures) . . . . .	104
XVII. The Stomach Oil of the Fulmar Petrel ( <i>Fulmarus glacialis</i> ). By O. ROSENHEIM and T. A. WEBSTER. (With one figure) . . . . .	111
XVIII. Studies on the Kinetics of Haemolytic Systems. III. Time-dilution Curves and Zones of Action. By E. PONDER. (With three figures) . . . . .	119

	PAGE
XIX. The Relation of Cholesterol to Vitamin D. By O. ROSENHEIM and T. A. WEBSTER . . . . .	127
XX. Studies on the Cholesterol Content of Normal Human Plasma. Part I. By J. A. GARDNER and H. GAINSBOROUGH . . . . .	130
XXI. Studies on the Cholesterol Content of Normal Human Plasma. Part II. The Attraction of the Proteins of Plasma for Sterols. By J. A. GARDNER and H. GAINSBOROUGH . . . . .	141
XXII. The Effects of Chemical and Physical Changes in Environment on Resting Bacteria. By J. H. QUASTEL and W. R. WOOLDRIDGE . . . . .	148
XXIII. Chemistry of Thyroxine. III. Constitution and Synthesis of Thyroxine. By C. R. HARRINGTON and G. BARGER. (With two figures.) With a Note by D. M. LYON . . . . .	169
XXIV. Echinochrome. By R. K. CANNAN . . . . .	184
XXV. The Inorganic Phosphate and a Labile Form of Organic Phosphate in the Gastrocnemius of the Frog. By P. EGGLETON and G. P. EGGLETON. (With two figures) . . . . .	190
XXVI. Note on the Preparation of Yeast-juice by Buchner's Method. By A. HARDEN and F. R. HENLEY . . . . .	196
XXVII. The Aerobic and Anaerobic Metabolism of the Common Cockroach ( <i>Periplaneta orientalis</i> ). II. By W. K. SLATER . . . . .	198
XXVIII. Some Phosphorus Compounds of Milk. III. Dephosphorised Caseinogen. The Action of Alkali upon Caseinogen. By C. RIMINGTON. (With one figure) . . . . .	204
XXIX. The Effect of Excessive Radiation with Ultra-violet Light upon the Growth of Rats. By J. L. LEIGH-CLARE . . . . .	208
XXX. A Further Note on the Antirachitic Value of Fresh Spinach. By M. H. ROSCOE . . . . .	211
XXXI. Optical Rotatory Power and Dispersion of Proteins. By L. F. HEWITT. (With three figures) . . . . .	216
XXXII. The Ether-soluble Substances of Cabbage Leaf Cytoplasm. I. Preparation and General Characters. By A. C. CHIBNALL and H. J. CHANNON . . . . .	225
XXXIII. The Ether-soluble Substances of Cabbage Leaf Cytoplasm. II. Calcium Salts of Glyceridephosphoric Acids. By A. C. CHIBNALL and H. J. CHANNON . . . . .	233
XXXIV. Analysis of Proteins. VIII. Estimation of Cystine in the Modified Van Slyke Method. By R. H. A. PLIMMER and J. LOWNDES . . . . .	247
XXXV. Changes in the Amino-acids in the Proteins of the Hen's Egg during Development. By R. H. A. PLIMMER and J. LOWNDES . . . . .	254
XXXVI. A Note on the Estimation of Chlorine in Milk. By A. D. HUSBAND and W. GODDEN . . . . .	259
XXXVII. The Bacterial Decomposition of Tobacco as Leading to the Formation of Bases in the Presence of Water. By A. FAITLOWITZ . . . . .	262

No. 2

	PAGE
OBITUARY NOTICE . . . . .	265
XXXVIII. The Use of the Hydroquinhydrone Electrode for $p_H$ Determination in the Fluids of the Organism. By F. GROSSMAN . . . . .	267
XXXIX. Phosphorylation of Proteins. By C. RIMINGTON. (With two figures) . . . . .	272
XL. The Dietary Value of Potato Protein. By G. A. HARTWELL. (With two figures) . . . . .	282
XLI. The Phosphorus Nucleus of Caseinogen. By S. POSTERNAK . . . . .	289
XLII. Contributions to the Study of Lignin. Part I. Metalignin, a New Type of Alkali Lignin. By C. DORÉE and E. C. BARTON-WRIGHT . . . . .	290
XLIII. Studies on Calcium Metabolism. I. The Action of the Parathyroid Hormone on the Calcium Content of the Serum and on the Absorption and Excretion of Calcium. By C. P. STEWART and G. H. PERCIVAL . . . . .	301
XLIV. On Liver Amylase. By G. S. EADIE. (With four figures) . . . . .	314
XLV. The Behaviour of Whole Blood towards Maltose <i>in vitro</i> . By A. HYND and M. G. MACFARLANE . . . . .	322
XLVI. Studies in the Metabolism of Tissues Growing <i>in vitro</i> . I. Ammonia and Urea Production by Kidney. By B. E. HOLMES and E. WATCHORN . . . . .	327
XLVII. The Catalytic Action of Traces of Iron and Copper on the Anaerobic Oxidation of Sulphydryl Compounds. By D. C. HARRISON . . . . .	335
XLVIII. Note on an Efficient Gas Scrubber. By H. W. SOUTHGATE. (With one figure) . . . . .	347
XLIX. Decomposition of Hexosephosphates by <i>B. coli communis</i> , Escherich. By R. J. MANNING . . . . .	349
L. A Note on the Precipitation of the Antiscorbutic Factor from Lemon Juice. By S. S. ZILVA . . . . .	354
LI. The Protein Error in Estimating $p_H$ with Neutral Red and Phenol Red. By E. H. LEPPER and C. J. MARTIN. (With three figures) . . . . .	356
LII. On the Absorption of Vitamin D from the Skin. By E. M. HUMR, N. S. LUCAS and H. H. SMITH . . . . .	362
LIII. A Search for Vitamin D in the Diatom <i>Nitzschia closterium</i> (W. Sm.). By J. L. LEIGH-CLARE . . . . .	368
LIV. The Carbohydrate and Fat Metabolism of Yeast. IV. The Nature of the Phospholipins. By C. G. DAUBNEY and I. SMEDLEY MACLEAN . . . . .	373
LV. Note on some Sterol Colour Reactions in their Relation to Vitamin A. By O. ROSENHEIM . . . . .	386
LVI. The Parent Substance of Vitamin D. By O. ROSENHEIM and T. A. WEBSTER. (With Plate I) . . . . .	389
LVII. The Influence of Amino-acids on Hydrolysis of Pancreatic Lipase. By E. R. DAWSON . . . . .	398
LVIII. The Micro-determination of Metals in Salts. By H. I. COOMBS. (With two figures) . . . . .	404
LIX. On the Alleged Action of X-rays upon Cholesterol. By I. HIEGER . . . . .	407



LX. Contributions to the Study of Brain Metabolism. IV. Carbohydrate Metabolism of the Brain Tissue of Depancreatized Cats. By B. E. HOLMES and E. G. HOLMES . . . . .	412
LXI. A Study of the Effect of Heat and Oxidation on Cod-liver Oil as Measured by Colour Tests. By F. WOKES and S. G. WILLIMOTT. (With one figure) . . . . .	419
LXII. A Note on the Ammonium Sulphate Precipitation of the Active Principle of the Culture Filtrates of <i>C. diphtheriae</i> . By A. F. WATSON and E. LANGSTAFF. (With two figures) . . . . .	426
LXIII. Unscaled Fibres. A New Aspect of Fibre Research. By A. T. KING. (With Plate II) . . . . .	434
LXIV. Note on the Effect of Light upon Uroporphyrin. By B. T. SQUIRES . . . . .	437
LXV. Influence of Glucose, Alcohol and Carbon Dioxide at Barometric Pressure on the $p_H$ Values of Phosphate and Bicarbonate Solutions, Determined by Means of Hydroquinhydrone Electrodes. By E. BILMANN and H. KATAGIRI. (With seven figures) . . . . .	441

## No. 3

LXVI. On the Photo-oxidation of Adrenaline. By T. VACEK. (With two figures) . . . . .	457
LXVII. Studies on the Growth of Yeast. IV. A Nephelometric Method of Counting Yeast Suspensions. By G. L. PESKETT. (With two figures) . . . . .	460
LXVIII. On the Isolation from Tissues of certain Pentose Derivatives. By L. B. WINTER . . . . .	467
LXIX. The Ether-soluble Substances of Cabbage Leaf Cytoplasm. III. The Fatty Acids. By A. C. CHIBNALL and H. J. CHANNON . . . . .	479
LXX. Observations on the Biochemistry of "Mutton Bird" Oil. By C. L. CARTER and J. MALCOLM . . . . .	484
LXXI. The Influence of the Fatty Acids and Hydroxy-acids and their Salts on Alcoholic Fermentation by Living Yeast. Part II. Propionic, Butyric, <i>iso</i> -Butyric, Glycollic, Lactic, Hydroxy- <i>iso</i> -butyric, $\alpha$ - and $\beta$ -Hydroxybutyric Acids and their Sodium Salts. By H. KATAGIRI. (With seven figures) . . . . .	494
LXXII. Oxidations by Hydrogen Peroxide in Presence of Sulphydryl Compounds. By D. C. HARRISON . . . . .	507
LXXIII. Experiments on the Formation of Succinic Acid in the Body. Part I. The Determination of Succinic Acid and its Formation in Muscle and Liver Pulp. By P. W. CLUTTERBUCK. (With two figures) . . . . .	512
LXXIV. Studies of the Urea Content of Capillary and Venous Blood. By E. SVENSGAARD . . . . .	522
LXXV. The Influence of Hexosediphosphoric Acid and Hexosemonophosphoric Acid upon Insulin Hypoglycaemia. By H. P. MARKS and W. T. J. MORGAN . . . . .	530
LXXVI. Haemocyanin. Part IV. The Dependence of the Shape of the Oxygen Dissociation Curve on the State of Ionisation of the Protein. By E. STEDMAN and E. STEDMAN. (With three figures) . . . . .	533
LXXVII. The Effect of Halogen Salts on Peptic Digestion. By W. M. CLIFFORD. (With one figure) . . . . .	544
LXXVIII. Studies in Carbohydrate Metabolism. III. The Influence of Dihydroxyacetone upon the Respiratory Metabolism and upon the Inorganic Phosphate of the Blood. By C. G. LAMBIE and F. A. REDHEAD . . . . .	549
LXXIX. The Purification and Properties of Insulin. By F. DICKENS, E. C. DODDS, W. LAWSON and N. F. MACLAGAN. (With one figure) . . . . .	560

# CONTENTS

ix

PAGE

LXXX. A Note on the Weight of the Rat during Gestation. By G. A. HAETWELL . . . . .	572
LXXXI. A Study of the System Carbonic Acid, Carbon Dioxide and Water. I. Determination of the True Dissociation-constant of Carbonic Acid. By F. J. J. BUYTENDYK, R. BRINKMAN and H. W. MOOK. (With four figures) . . . . .	576
LXXXII. On the Development of Chromogenic Properties in Cholesterol by the Action of Heat. By T. MOORE and S. G. WILLIMOTT . . . . .	585
LXXXIII. The $p_H$ of the Gastro-intestinal Tract of Certain Rodents used in Feeding Experiments, and its Possible Significance in Rickets. By T. REDMAN, S. G. WILLIMOTT and F. WOKES. (With five figures) . . . . .	589
LXXXIV. Studies in the Sulphur Metabolism of the Dog. V. Toxic Action of Mercapturic Acids. By E. H. CALLOW and T. S. HELE. (With three figures) . . . . .	606
LXXXV. Studies in the Sulphur Metabolism of the Dog. VI. The Metabolism of the Pig and Dog compared. By H. I. COOMBS and T. S. HELE. (With two figures) . . . . .	611
LXXXVI. Studies in the Sulphur Metabolism of the Dog. VII. The Effect of Fluorobenzene on Sulphur Metabolism. By H. I. COOMBS. (With two figures) . . . . .	623
LXXXVII. The Reduction of Haematin and Methaemoglobin. By R. HILL and H. F. HOLDEN. (With one figure) . . . . .	625
LXXXVIII. The Physiological Rôle of Vitamin B. Part III. Study of Vitamin B Deficiency in Pigeons. By S. K. KON and J. C. DRUMMOND. (With Plates III, IV and four figures) . . . . .	632
LXXXIX. The Physiological Rôle of Vitamin B. Part IV. The Relation of Certain Dietary Factors in Yeast to Growth of Rats on Diets Rich in Proteins. By A. HASSAN and J. C. DRUMMOND. (With two figures) . . . . .	653
XC. A Conductivity Method for the Determination of Carbon Dioxide. By L. E. BAYLISS. (With one figure) . . . . .	662
XCI. The Possible Significance of Hexosephosphoric Esters in Ossification. Part VII. The Bone Phosphatase. By M. MARTLAND and R. ROBISON. (With four figures) . . . . .	665
XCII. The Chemistry of Hexosediphosphoric Acid. Part I. The $\alpha$ - and $\beta$ -Methyl-hexosediphosphoric Acids. By W. T. J. MORGAN. (With three figures) . . . . .	675
XCIII. The Antiscorbutic Fraction of Lemon Juice. V. By S. S. ZILVA . . . . .	689
XCIV. On the Composite Nature of the Water-soluble B Vitamin. By H. CHICK and M. H. ROSCOE. (With two figures) . . . . .	698
XCV. The Effect of Desiccation upon the Nutritive Properties of Egg-white. By M. A. BOAS. (With Plate V and four figures) . . . . .	712
XCVI. A Note on the Vitamin D Content of the Stomach Oil of the Australasian Petrel ( <i>Australa Lessoni</i> ). By J. L. LEIGH-CLARE . . . . .	725
XCVII. The Synthesis of the Antineuritic Factor (Torulin) by Yeast. By F. HAWKING . . . . .	728
XCVIII. On Ovomucoid. By J. NEEDHAM . . . . .	733
XCIX. A Quantitative Study of Succinic Acid in Muscle. II. The Metabolic Relationships of Succinic, Malic and Fumaric Acids. By D. M. NEEDHAM. (With two figures) . . . . .	739
C. Quantitative Studies of the Nitroprusside Reaction in Normal Tissues and Tumours. By E. L. KENNAWAY and I. HIEGER . . . . .	751
CI. Observations on the Renal Threshold for Glucose. By R. L. MACKAY. (With one figure) . . . . .	760

# CONTENTS

## No. 4

### PAGE

CII. The Enzymic Production of Volatile Products from Nicotine under the Influence of Tobacco-leaf Extracts. (A Reply to A. Faitelowitz.) By A. FODOR and A. REIFENBERG . . . . .	765
CIII. The Interaction of a Finely Divided Lead Suspension with Blood-serum, Ringer Solution, and Aqueous Phosphate Solution. By J. BROOKS. (With two figures) . . . . .	766
CIV. Antineuritic Yeast Concentrates. II. The Use of Norite Charcoal in the Concentration of Torulin. By H. W. KINNERSLEY and R. A. PETERS . . . . .	777
CV. Digestion in the Cockroach. I. The Hydrogen Ion Concentration in the Alimentary Canal. By V. B. WIGGLESWORTH. (With one figure) . . . . .	791
CVI. Digestion in the Cockroach. II. The Digestion of Carbohydrates. By V. B. WIGGLESWORTH. (With nine figures) . . . . .	797
CVII. The Production of Lactic Acid in Frog's Muscle <i>in vivo</i> . By C. E. WOODROW and V. B. WIGGLESWORTH . . . . .	812
CVIII. The Titration of Protein Hydrolysates. By W. L. DAVIES . . . . .	815
CIX. Variations in the $p_H$ and Bicarbonate of the Plasma and of the Alveolar $CO_2$ during Forced Breathing. By E. H. LEPPER and M. MARTLAND. (With four figures) . . . . .	823
CX. The Influence of Meals on the Rise of the Hydrogen Ion Concentration of the Blood during Hyperpnoea. By E. H. LEPPER and M. MARTLAND. (With two figures) . . . . .	831
CXI. A Note on the Temporary Spontaneous Disappearance of Typical "Beriberi" Symptoms in Pigeons Fed on Diets Deficient in Vitamin B. By S. K. KON . . . . .	834
CXII. The Effect of the Administration of Glycine to Pigeons on a Diet Deficient in Vitamin B. By S. K. KON . . . . .	837
CXIII. The Effect of Cyanide on the Schardinger Enzyme. By M. DIXON. (With two figures) . . . . .	840
CXIV. On the Reducing Power of Glutathione and Cysteine. By M. DIXON and H. E. TUNNICLIFFE. (With one figure) . . . . .	844
CXV. Note on the Erlenmeyer Amino-acid Synthesis. By C. R. HARRINGTON and W. MCCARTNEY . . . . .	852
CXVI. The Enzymic Deamidation of Asparagine in the Higher Plants. By C. E. GROVER and A. C. CHIBNALL. (With two figures) . . . . .	857
CXVII. Note on the Unsaponifiable Matter of Yeast-fat. By C. G. DAUBNEY and I. SMEDLEY MACLEAN . . . . .	869
CXVIII. Vitamin A Formation in the Etiolated Wheat Shoot. By T. MOORE. (With one figure) . . . . .	870
CXIX. The Influence of Temperature on the Nature of the Fat formed by Living Organisms. By L. K. PEARSON and H. S. RAPER . . . . .	875
CXX. The Glucose Metabolism of Kidney Tissue <i>in vitro</i> . I. By J. T. IRVING. (With two figures) . . . . .	880
CXXI. Vitamins A and D of Spinach. By S. G. WILLIMOTT and F. WOKES. (With one figure) . . . . .	887
CXXII. On the Early Stages of Microbiological Decay and Humification of Vegetable Tissues. By A. C. THAYSEN and W. E. BAKES . . . . .	895

# CONTENTS

xi

PAGE

CXXXIII. The Relation of the Growth of Certain Micro-organisms to the Composition of the Medium. I. The Synthetic Culture Medium. By V. READER	901
CXXXIV. The Relation of the Growth of Certain Micro-organisms to the Composition of the Medium. II. The Effect of Changes of Surface-tension on Growth. By V. READER. (With one figure)	908
CXXXV. Experiments on Nutrition. VI. Balance of Food by Vitamin B. By R. H. A. PLIMMER, J. L. ROSEDALE and W. H. RAYMOND. (With Plate VI)	913
CXXXVI. Experiments on Nutrition. VII. Fat-soluble Vitamin Requirements of Chickens. By R. H. A. PLIMMER, J. L. ROSEDALE and W. H. RAYMOND	940
CXXXVII. The Diurnal Variation of the Gaseous Constituents of River Waters. By R. W. BUTCHER, F. T. K. PENTLOW and J. W. A. WOODLEY. (With four figures)	945
CXXXVIII. Blood-fat and Exercise. By J. W. T. PATERSON. (With two figures)	958
CXXXIX. The Dispersed Phase of the Blood Corpuscles. By R. EGE	967
CXXX. The Influence of Variations in the Sodium-Potassium Ratio on the Nitrogen and Mineral Metabolism of the Growing Pig. II. By M. B. RICHARDS, W. GODDEN and A. D. HUSBAND. (With two figures)	971
CXXXI. The Toxicity of Phenylarsinic Acids for <i>Balantidium coli</i> Malm. in Cultures, in Relation to their Chemical Constitution. By A. S. CORBET and A. P. JAMESON	986
CXXXII. The Effect of Alkalosis on the Excretion of Chloride and on Carbohydrate Metabolism. By M. W. GOLDBLATT	991
CXXXIII. A Note on the Estimation of Adrenaline. By L. C. BAKER and G. F. MARRIAN. (With two figures)	1005
CXXXIV. The Constituents of <i>Fabiana imbricata</i> . By G. R. EDWARDS and H. ROGERSON	1010
CXXXV. Variations in the Serum-calcium of Rabbits. By K. CULHANE. (With five figures)	1015

## No. 5

CXXXVI. Structure and Enzyme Reactions. Parts I and II. The Systems Urea-urease-charcoal and Polysaccharide-amylase-charcoal. By ST. J. PRZYLECKI, H. NIEDZWIEDZKA and T. MAJEWSKI	1025
CXXXVII. Studies in Purine Metabolism. III. Basal Metabolism and Purine Content. By R. TRUSZKOWSKI	1040
CXXXVIII. Studies in Purine Metabolism. IV. The Nuclear-plasmic Ratio in Dogs in Carbohydrate and Protein Feeding, and in Starvation. By R. TRUSZKOWSKI	1047
CXXXIX. Reaction of Fatty Extracts of Certain Organs with the Antimony Trichloride Test for "Vitamin A." (Preliminary Note.) By W. H. WILSON	1054
CXL. On the Pigment Produced by <i>Chromobacterium violaceum</i> . By J. REILLY and G. PYNE	1059
CXLI. The Preparation of Taurine in Considerable Quantity. By W. O. KERMACK and R. H. SLATER	1065

CXLII. Carbon Monoxide as a Tissue Poison. By J. B. S. HALDANE. (With one figure) . . . . .	1068
CXLIII. Growth and Reproduction on Synthetic Diets. II. By G. A. HARTWELL. (With three figures) . . . . .	1076
CXLIV. The Photosynthesis of Urea from Ammonium Carbonate. By W. R. FEARON and C. B. M'KENNA . . . . .	1087
CXLV. The Effect of Certain Sugar Derivatives on Insulinised Mice. By A. HYND . . . . .	1091
CXLVI. The Antagonism of Glucosone and Cyanides <i>in vivo</i> . By A. HYND	1094
CXLVII. The Synthesis of Antineuritic Vitamin by Yeast. By G. L. PESKETT . . . . .	1102
CXLVIII. Kidney Phosphatase. II. The Enzyme in Disease. By R. T. BRAIN and H. D. KAY . . . . .	1104
CXLIX. Identity of Urinary Albumin. By L. F. HEWITT . . . . .	1109
CL. The Ether-soluble Substances of Cabbage Leaf Cytoplasm. IV. Further Observations on Diglyceridephosphoric Acid. By H. J. CHANNON and A. C. CHIBNALL . . . . .	1112
CLI. The Antiscorbutic Fraction of Lemon Juice. VI. By E. HOYLE and S. S. ZILVA . . . . .	1121
CLII. Liver Diastase. By E. F. LESSER . . . . .	1128
CLIII. The Effect of Hydrogen Ion Concentration and Protein Concentra- tion on the Osmotic Pressure of Serum-proteins. By J. MARRACK and L. F. HEWITT. (With three figures) . . . . .	1129
CLIV. Experiments on Nutrition. VIII. Comparative Vitamin B Value of Foodstuffs. Cereals. I. By R. H. A. PLIMMER, J. L. ROSEDALE, W. H. RAYMOND and J. LOWNDES . . . . .	1141
CLV. The Estimation of Cyanogenetic Glucosides. By L. R. BISHOP. (With one figure) . . . . .	1162
CLVI. Neutral Salt Addition Compounds of Alkaline Earth Glutamates and Aspartates. By W. K. ANSLOW and H. KING. (With two figures) . . . . .	1168
CLVII. The Phosphorus of Caseinogen. I. Isolation of a Phosphorus- containing Peptone from Tryptic Digests of Caseinogen. By C. RIMINGTON . . . . .	1179
CLVIII. The Phosphorus of Caseinogen. II. Constitution of Phospho- peptone. By C. RIMINGTON. (With one figure) . . . . .	1187
CLIX. Observations on some Processes of Oxidation in Blood-serum. By T. R. PARSONS and W. PARSONS. (With nine figures) . . . . .	1194
CLX. The Specific Action of Plant Enzymes. III. The Specific Conditions of Action of Leaf Salicinascs. By A. V. BLAGOVESCHENSKI and N. I. SOSSIEDOV	1206
CLXI. The Action of Carbon Monoxide on Certain Oxidising Enzymes. By M. DIXON. (With three figures) . . . . .	1211
CLXII. The Equation of Alcoholic Fermentation. By A. HARDEN and F. R. HENLEY . . . . .	1216
CLXIII. Experiments on Bacteria in relation to the Mechanism of Enzyme Action. By J. H. QUASTEL and W. R. WOOLDRIDGE. (With three figures) . . . . .	1224

No. 6

	PAGE
CLXIV. The Photocapillary Reaction of Plant Sap. By F. HERŐTK. (With two figures) . . . . .	1253
CLXV. Studies on Xanthine Oxidase. IX. The Specificity of the System. II. By H. I. COOMBS. (With one figure) . . . . .	1259
CLXVI. Studies on the Antineuritic Vitamin. I. Preliminary Note on a Possible Second Factor. By J. L. ROSEDALE . . . . .	1266
CLXVII. The Effects of Cod-liver Oil on the Calcium and Phosphorus Metabolism of the Lactating Animal. By D. HARVEY. (With two figures) . . . . .	1268
CLXVIII. The Glutathione Content of Chicken Sarcoma: A Rejoinder. By H. YAOI and W. NAKAHARA . . . . .	1277
CLXIX. The Absorption Spectra of Oils and Oil Constituents with Special Reference to Pro-vitamin D. By I. M. HEILBRON, E. D. KAMM and R. A. MORTON. (With five figures) . . . . .	1279
CLXX. Investigations on Gelatin. Part IX. The Scission of Gelatin into Constituent Proteins. By S. B. SCHRYVER and K. V. THIMANN. (With one figure) . . . . .	1284
CLXXI. Investigations on Gelatin. Part X. The Effect of Previous Treatment with Acid and Alkali on the Diamino-nitrogen Fraction of the Hydrolysis Products of Gelatin. By B. THORNLEY. (With one figure) . . . . .	1302
CLXXII. Combination of Proteins with Phthalein Dyes. By L. F. HEWITT . . . . .	1305
CLXXIII. The Surface Tension of Protein Solutions. Part III. By J. H. ST JOHNSTON. (With two figures) . . . . .	1314
CLXXIV. A New Colorimeter Based on the Lovibond Colour System, and its Application to the testing of Cod-liver Oil, and other Purposes. By O. ROSENHEIM, with an Addendum by E. SCHUSTER. (With two figures) . . . . .	1329
CLXXV. Note on the Induced Fluorescence of Ergosterol. By O. ROSENHEIM . . . . .	1335
CLXXVI. The Physiological Rôle of Vitamin B. Part V. The Relation of Inanition to Vitamin B Deficiency in Pigeons. By G. F. MARRIAN, L. C. BAKER, J. C. DRUMMOND and H. WOOLLARD. (With two figures) . . . . .	1336
CLXXVII. A Note on the Effects on Pigeons of an Exclusive Diet of Rice Meal, Bran and Polish. By R. R. WILLIAMS . . . . .	1349
CLXXVIII. The Absorption of Water by Gelatin. By D. JORDAN LLOYD and W. B. PLEASS. (With seven figures) . . . . .	1352
CLXXIX. Note on the Attempted Activation of Tyrosine by Ultra-violet Irradiation. By S. K. KON and T. MOORE . . . . .	1368
CLXXX. The Action of Tyrosinase on Phenols. With some Observations on the Classification of Oxidases. By C. E. M. PUGH and H. S. RAPER . . . . .	1370
CLXXXI. Dissociation Constants of Cystine, Cysteine, Thioglycollic Acid and $\alpha$ -Thiolactic Acid. By R. K. CANNAN and B. C. J. G. KNIGHT. (With one figure) . . . . .	1384
CLXXXII. Studies in the Metabolism of Tissues Growing <i>in vitro</i> . II. Effect of Glucose upon the Ammonia and Urea Production of Kidney Tissue. By E. WATCHORN and B. E. HOLMES . . . . .	1391
CLXXXIII. The Effect of Adrenaline on Ketosis in Phloridzinised and Normal Rats. By A. B. ANDERSON and M. D. ANDERSON. (With six figures) . . . . .	1398

	PAGE
CLXXXIV. The Autocatalytic Oxidation of Sulphydryl Compounds. By D. C. HARRISON. (With one figure) . . . . .	1404
CLXXXV. The use of the Starch-iodine End-point in Tunncliffe's Method for the Determination of Glutathione in Tissues. By W. A. PERLZWEIG and G. DELRUE . . . . .	1416
CLXXXVI. A Note on the Hypoglycaemic Action of Dihydroxyacetone in Man. By E. P. CATHCART and J. MARKOWITZ. (With one figure) . . .	1419
CLXXXVII. The Diurnal Variation of the Gaseous Constituents of River Waters. Part II. By R. W. BUTCHER, F. T. K. PENTELOW and J. W. A. WOODLEY. (With six figures) . . . . .	1423
INDEX . . . . .	1437

# I. A STUDY OF THE VARIATIONS IN THE CHEMICAL COMPOSITION OF NORMAL HUMAN COLOSTRUM AND EARLY MILK.

By MARGARET FRANCES LOWENFELD (*Alfred Langton Research Scholar*),  
SIBYL TAITE WIDDOWS, MURIEL BOND  
AND EFFIE ISABEL TAYLOR.

*From the Obstetric Unit of the Royal Free Hospital and the London School  
of Medicine for Women.*

(Received October 25th, 1926.)

A VERY extensive literature has grown up during the past half century, on the subject of the chemistry of normal human milk. So vast has this literature become, that the task of absorbing and correlating its conclusions into a coherent understanding of the subject as a whole has become one of extreme difficulty and laboriousness. On the other hand, some attempt to do so must form the basis of any new work. From a review of the main communications in the literature, a list of which is appended, certain facts stand out and can, we suggest, be made the basis of an attempt to explore the matter in greater detail.

1. We have reliable and accurate knowledge of the general nature of the main constituents of human milk, and adequate methods for their estimation.

2. We have on record a very large number of observations of milk at all stages of lactation, and are aware of the changes most usually found.

If, however, we review the aggregate of facts presented by points 1 and 2, an admirable consideration of which appeared in a communication last year [Gardner and Fox, 1925], the main feature of the picture is the exceedingly wide range of values shown in normal milk by each constituent. These variations complicate our understanding of the composition of normal milk, and put a serious bar to any study of the conditions of abnormal milk. Furthermore, the great bulk of the work done hitherto on normal milk has not only been done abroad, but has also been achieved through the study of wet nurses—that is, of women accustomed to a specialised routine for the production and extraction of milk. As their conditions differ radically from those of ordinary nursing mothers, it cannot safely be assumed that the lactation results will necessarily be the same.

The present paper is the report of an attempt on the basis of work already known to deal with both these positions, to investigate in ordinary normal mothers the factors determining the variations in the chemical composition of their milk.



## METHOD AND MATERIAL.

The period of lactation chosen for investigation was the first 14 days after parturition, the reason for this choice being the accessibility to continuous observation of women in a labour ward, and the scantiness of our knowledge of this period. The mothers selected were all normal women, in-patients of the Obstetric Unit of the Royal Free Hospital. Among these patients were some who had been admitted to the V.D. Section of the Obstetric Unit for gonorrhoea. No mother with a history of syphilis was included.

In the vast majority of previously published communications the milk for analysis was obtained from single samples of the milk of different women. As by this procedure no opportunity is given for the observation of individual differences or their bearing upon general results, our procedure has been instead to make continuous observations of a number of individual women throughout their stay in the maternity wards.

Every sample was collected personally in the wards by one of us (M. F. L.), and taken to the London School of Medicine for Women where the analyses were carried through on the same day by S. T. W., M. B. and E. I. T. In only two instances was the milk kept overnight.

*Chemical methods.*

The constituents examined were protein, sugar, ash, fat, calcium and solids. Our aim at each point has been, not so much to achieve absolute percentages, as to arrive at some idea of the grounds underlying the differences that we found. The following are the methods of chemical estimation employed.

*Protein.* The total nitrogen was estimated by the Kjeldahl-Gunning method. 2.5 cc. of milk were usually taken for analysis, and the protein precipitated by saturated tannic acid solution, but where the amounts of milk available for examination were small, 1.5 cc. or occasionally 1 cc. only was used for the determination of N. In all cases where possible, the estimations have been done in duplicate with appropriate blank experiments.

*Sugar.* The method of Folin and Denis [1918] was employed. Calculation of the sugar content from the figures obtained from titration is according to the following empirical formula given by Folin and Denis:

$$\frac{4.04 \times \text{dilution}}{\text{titration}}$$

*Fat.* The method of Gottlieb [1892] has been used, 5 cc. of milk being usually taken; but where the amount of milk available did not allow of this quantity, the maximum amount that could be given was diluted to 5 cc. with water before extraction of fat. In all these estimations the term "fat" is used to denote all the ether-extractable substances.

*Solids and ash.* 2.5 cc. milk were measured into a weighed platinum basin; the fluid was evaporated on a water-bath for half-an-hour and then transferred to a steam-oven and heated to constant weight. The solid residue was converted into ash by ignition at low temperature, the basin never being allowed

to show a red glow. In some cases a duplicate estimation was made by just charring the solid residue, dissolving out the salts with water and filtering through a small tared filter. The carbon and filter paper after drying were ignited together, the filtrate added, evaporated to dryness and gently ignited. No difference in result was obtained by these two methods.

*Calcium.* A modification of the McCrudden [1912] method for small quantities of calcium, as elaborated for the estimation of calcium in blood [Widdows, 1923] was adopted. All the precautions which were taken in the estimation of calcium in blood serum were also observed here. In addition the milk was diluted (1 in 10) before use for estimation, to render the percentage of calcium comparable with that of serum. The results were calculated to g. of calcium oxide per 100 cc.

#### *Clinical methods.*

Very early in the investigation it became quite clear that the method of extraction of the milk had a profound bearing upon the composition of the sample obtained. No uniform technique was therefore adhered to, but the method was varied in each instance according to the problem under consideration. A note of the technique employed, therefore, accompanies each group of figures, the symbol *P* being equivalent to the use of a breast pump, *H* signifying digital expression. For the main part, and unless otherwise stated, the samples were collected between 10 a.m. and 11 a.m. and in immediate relation to the 10 a.m. or 10.30 a.m. feed. Altogether 164 samples were investigated—wholly or partly—of 13 women. That is, 5 women through 8–13 days and 8 women through 2–6 days.

#### RESULTS.

It has been the custom in most papers of this kind to print the values, found for all the constituents of the milks examined, in a single table. By this means, the exact composition of each sample of milk can be seen. However, it has been found that the behaviour of the different constituents in the results to be demonstrated could not be clearly shown unless they were considered separately. The figures in the column headed No. are the laboratory labels of the sample of milk from which the specimen was taken and afford a means of correlation between the various tables. Each of the constituents observed will therefore be examined in order, according to its behaviour during the period under observation and its reaction to circumstances local to the taking of the individual sample of milk.

*Protein.* In the consideration of the protein content of the milk, 4 women were studied for 11 days, and 8 women for 4–8 days, and Fig. 1 represents the values found. The curves for *primiparae* are shown in an unbroken, and those for *multiplarae* in a broken line.

It will be seen that in every case the highest protein percentage is to be found in the first 3 days after parturition, and that the level falls steeply during the first week to reach an approximate average of 1.4 % by the end

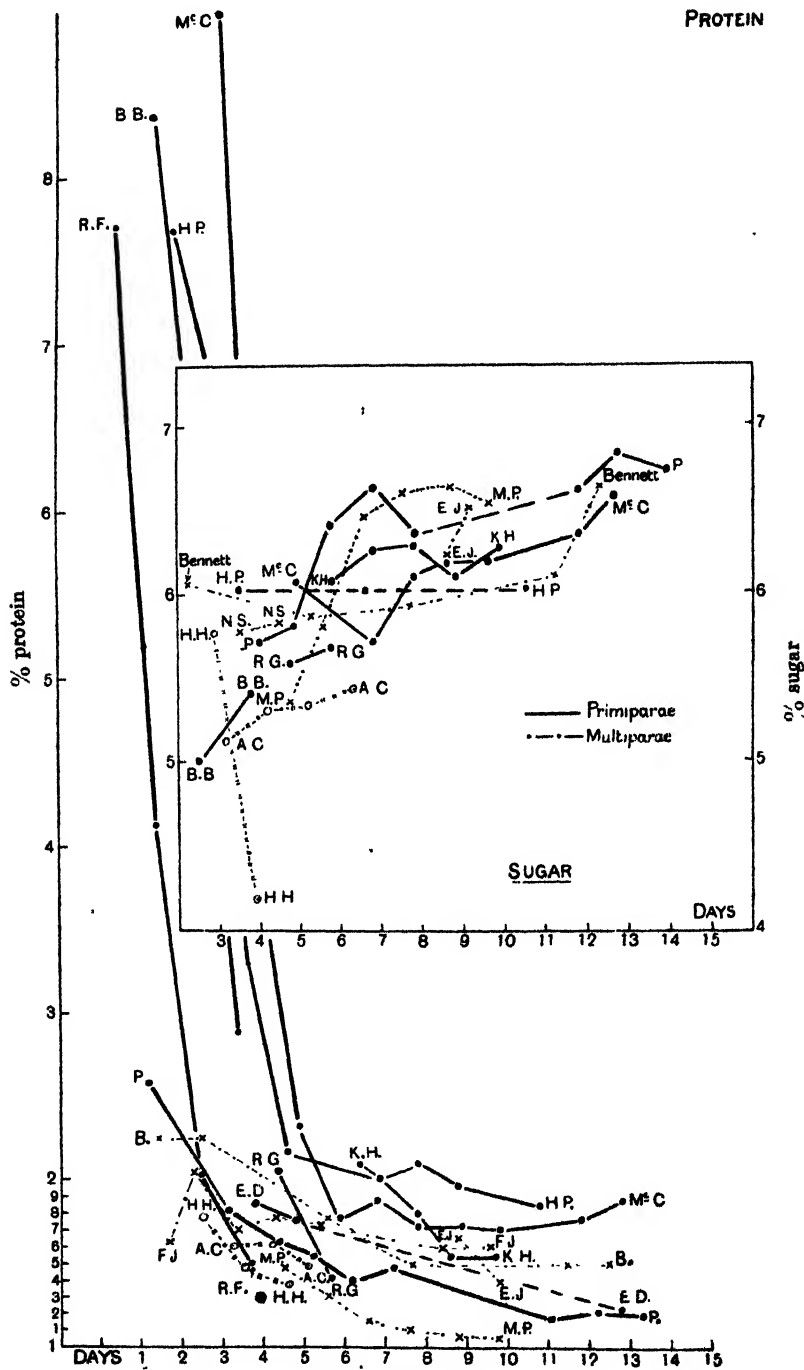


Fig. 1.

of the 13th day. F. J. is the one exception. It may further be observed that as far as this small number of cases is concerned the initial protein values for *primiparae* appear to be much higher than those for *multiparae*. Moreover the curves followed by the *multiparae* show a quicker fall than those of the *primiparae* and reach a lower constant level. These observations are borne out by the protein values given by Adriance [1897] and Carter and Richmond [1898] if their tables are arranged in this way.

It is also possible that the quantity of milk available for the sample may be of significance in modifying the percentage of protein in it. The volumes obtained for McC., B. B. and H. P., by expression or when the amount taken by the baby (ascertained by test weighing) is considered in addition, do not exceed 12 cc., whereas with F. J. and M. P. the minimum amount obtained was 30 cc. and rose several times to 60 cc. or 90 cc. On the other hand, a percentage of 2.9 has been found on the 5th day in a sample of 105 cc. and 1.74 in a sample of 9.7 cc. It would appear therefore that the total volume of fluid in the breast may correlate with the percentage of protein found in the milk though this consideration, as is shown later, does not seem to influence the sugar or the calcium. It is worthy of note also that in most cases it is those individuals who show a high protein content in their early milk who have also a high value for total ash.

*Sugar.* If the sugar values for individual women are plotted in a graph in the same manner as those for protein, a similarly definite relationship between them becomes apparent. This, within certain limits, appears to be constant for all women. That is to say, that apart from any other consideration, the day of lactation upon which a sample of milk is taken will have a marked and definite relationship to the amount of sugar to be found in the milk. The graph (Fig. 1) represents the course taken by the sugar values of 11 cases through 2-11 days. It will be noticed that the lowest values are to be found in the early days of lactation and that these rise irregularly throughout the first 14 days. The direction of the curve taken is, however, opposite to that of protein. No such very low sugars were found by us as by some other observers, our lowest percentage exclusive of case H. H., whose other constituents also showed abnormalities, falling only to 4.5 %. Unfortunately, no sample for sugar estimation was obtained earlier than the 3rd day after parturition.

As regards comparison between the milk of *multiparae* and *primiparae*, although it would appear possible on the whole that that of *primiparae* shows a somewhat lower sugar value than that of *multiparae*, yet no constant rule is observable for this.

*Ash.* After as large a quantity of milk had been obtained at a single feed as the circumstances would permit, such estimations of the ash were carried out upon it as the volume would allow. For the purpose of satisfactory ash analysis the collection of the whole 24 hours' milk is strongly advised by Holt, Courtney and Fales [1915], but, as our interest was in the development of the

mineral content in individual women through a period of days, rather than in the exact constitution of the ash itself, this procedure could not be adopted. The quantity of milk upon which the analysis was done was in most cases 2.5 cc., though in a few cases a somewhat smaller volume had to be taken. Altogether 73 samples of 9 women were investigated representing 2 *multiparae* and 7 *primiparae*. The values obtained are represented in Fig. 2, plotted according to the average results for each day obtained for each individual for the period under observation.

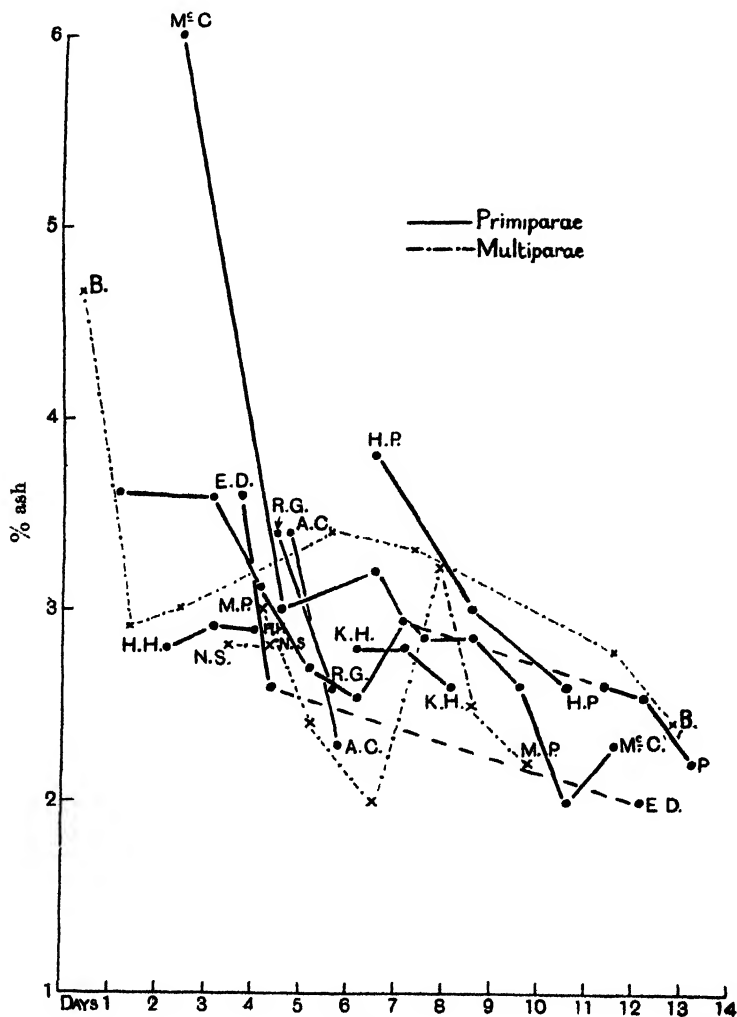


Fig. 2.

It will be seen that the course of the mineral constituents tends to show the same type of variation as that of protein and sugar, the direction of the curve, however, is analogous with that of the protein. Unfortunately the

number of samples of the milk of *multiparae* is very small and therefore no true comparison can be instituted between the curve of these and the *primiparae*. The tendency, however, seems to be in accordance with that of protein, that is, that the higher percentages appear in the early milk of *primiparae* rather than of *multiparae*. The fall is by no means so regular as that of the protein, and there are certain unexplained swings to be noticed in many curves. In the one example of continuous work on early ash found in the literature [Woodward, 1897], the same swinging values are found, although the figures are on the whole lower than our percentages. All the other analyses are unfortunately of single specimens of different women and so not susceptible of comparison.

The next point for general consideration is the question of the possible variation in the composition of milk during the course of a single feed. As a means of investigating this point the following two procedures were adopted. In the first, a sample of milk was taken (either by hand or with a breast pump) before the child was fed, the child was then put to the breast for a varying period of time and finally a sample was taken by hand or pump after it had sucked. By the alternative method the baby was allowed to take his whole feed from the one side, while an equal or larger amount was taken simultaneously or immediately after from the other and analysed in fractional portions, the "first" and "third" of these corresponding to the "before" and "after" of the other method. The composition of the milk of either sample was investigated with regard to the percentage content in it of each constituent in turn. Table I represents these values for protein (*i.e.* N  $\times$  6.37) and sugar in percentages.

Table I. *Variations in single feed. Protein and sugar.*

With baby						Without baby							
No.	Sample in cc.		Before		After		No.	Sample in cc.		First		Last	
			Sugar	Protein	Sugar	Protein				Sugar	Protein	Sugar	Protein
16/ 17	30	30	5.47 <i>P</i>	2.05 <i>P</i>	5.70 <i>P</i>	2.20 <i>P</i>	30/ 31	21	27	5.31 <i>P</i>	1.51 <i>P</i>	5.46 <i>P</i>	1.50 <i>P</i>
88/ 89	6.3	6.3	4.93	1.95	5.62	2.14	33/ 34	26	26	5.47 <i>P</i>	1.31 <i>P</i>	5.49 <i>H</i>	1.33 <i>H</i>
141/142	22	25	5.04	1.60 <i>P</i>	5.77	1.70 <i>P</i>	45/ 47	29	29	6.78 <i>Dr</i>	0.96 <i>Dr</i>	6.39 <i>H</i>	0.96 <i>H</i>
143/144	30	15	5.94 <i>P</i>	1.60 <i>P</i>	5.68 <i>P</i>	1.74 <i>P</i>	116/117	55	25	5.95 <i>P</i>	1.53 <i>P</i>	5.56 <i>P</i>	1.53 <i>P</i>
86/ 87	2.6	6.3	—	1.78	—	2.09	119/120	27	30	4.08 <i>P</i>	1.37 <i>P</i>	4.45 <i>P</i>	1.37 <i>P</i>
138/139	5.0	5.5	—	2.50 <i>H</i>	—	2.67 <i>H</i>	160/161	22	40	6.90 <i>P</i>	1.14 <i>P</i>	6.80 <i>H</i>	1.23 <i>H</i>
146/147	23	24	—	1.49	—	1.33	168/169	3	11	5.64 <i>H</i>	2.24 <i>H</i>	5.65 <i>H</i>	2.24 <i>H</i>
149/150	22	12	—	1.57	—	1.43	174/176	53	30	6.56 <i>H</i>	1.21 <i>H</i>	6.65 <i>H</i>	1.21 <i>H</i>
126/127	6	6	6.11 <i>H</i>	—	5.31 <i>H</i>	—	40/ 42	30	30	6.53 <i>H</i>	—	6.53 <i>H</i>	—
134/135	9.5	11	6.40 <i>H</i>	—	5.90 <i>H</i>	—	49/ 51	30	31	—	1.12 <i>Dr</i>	—	1.12 <i>H</i>
186/187	20	11	—	1.42	—	1.49	111/112	25	13	5.54 <i>P</i>	—	5.44 <i>P</i>	—
152/153	14	8	7.11 <i>P</i>	—	6.14 <i>P</i>	—	101/102	12	20	—	1.16 <i>Dr</i>	—	1.16 <i>P</i>

As regards the *protein* it will be seen that in the columns representing the values found before and after a feed there is a larger range of difference than in that of the milk obtained from a complete emptying of one breast, the highest being 0.31 % which represents approximately 14 % of the total value found. On the other hand, in the latter case practically all the samples are stationary since differences of 0.01 % and 0.02 % are, in this case, within

the limit of experimental error. If any constant variation therefore can be said to occur in the protein, it is in the direction of a rise in the "after" specimen.

With regard to the *sugar* it will be noticed that in the section "With baby," 5 show a decrease, 2 an increase, and none is stationary. In the other section, 4 show a decrease, 3 show an increase and 3 are stationary. Both the more frequent and, with one exception, the maximum variations it will be seen are in the downward direction, which is in line with the suggestion already made by Denis and Talbot in this matter [1919], that the sugar content of the milk of the first part of a feed is higher than that of the last. This is to be seen most clearly in the dripped samples (*Dr*). The method of extraction of the milk appears to have no influence upon the sugar values.

With regard to the *comparison of samples taken from either breast*, under as nearly as possible similar conditions, out of 12 samples examined for protein, 6 showed no difference, and the difference in the others (0.1 % to 0.2 %) when taken in association with the fact that the sample from the one breast was of the whole quantity, and the other from the sum of that taken before and after feeding, is probably relative to the variation already considered between the early and late milk of a single feed. With regard to the sugar content, in those instances in which both breasts, at the time at which the sample was taken, were entirely normal, no difference appeared.

As concerns the *ash* with regard to the variations in a single feed and the comparison of the milk of either side, such differences as were found to exist do not follow any rule. The number of our examples here, however, is too small to be of significance.

*Fat*. It has been seen that protein, sugar and ash show variations in percentage content according to the day of lactation. With fat this factor is not operative. If a list of fat values for a number of cases be written down in sequence, according to the date after parturition, no order is discoverable in their values. In the cases considered some milks showed 1 % fat on the 4th day and 7 % on the 7th, or 7 % on the 2nd and 2 % on the 11th. There were *primiparae* who showed a range of 0.6 % to 2 % and *multiparae* giving 2.9 % to 5.9 %. But if, on the other hand, instead of the day of lactation, the variations in a single feed come to be considered, a consistent picture develops.

In representing the amounts taken by the baby, in certain instances, as for example No. 84/85, only the total amount taken from both breasts was known, and as for the purpose of this table only one breast was relevant and the exact amount taken from that side is not discoverable, the quantity, therefore, taken by the baby is represented by the total amount from both sides divided by 2.

In Table II the following points will be noticed.

1. There is a rise in fat content in all samples but one, and this rise in all cases, other than those specially marked, is more than 1 % and can reach

as high as 5.8 %. If moreover, omitting the cases starred, the average be taken of the differences in the section "With baby" it will be found to be higher than that of the first and last samples.

2. The greatest difference in both sections appears in those cases (marked *Dr* and *H*) where the first sample was of milk that had dripped from one breast while the baby fed from the other, and the last sample was taken by hand.

3. In all cases but four (marked \*) the method of procedure was either to take the first sample with a breast pump and to finish by digital expression, or to use the same technique for the collection of both samples. In examples 78/79, 88/89, 156/157 and 172/173 the usual procedure was reversed, the first part of the specimen being taken by hand and the last by pump. In the first three of these the result has been to reduce the difference normal for these individuals (1.1 %, 5.4 % and 1.3 % respectively) to 0.2 %, 0.6 % and 0.4 %, whilst in case 172/173 it has fallen to - 0.6.

Table II. *Variations in single feed. Fat (%)*.

With baby									Without baby							
No.	Sample in cc.		Preg.	Day	Before	Ozs. taken by baby	After	Difference	No.	Sample in cc.		Preg.	Day	First	Last	Difference
186/187	20	11	P	4	1.52 <i>HP</i>	2.25	2.88 <i>HP</i>	+1.3	116/117	55	25	P	4	1.62 P	2.83 P	+1.2
141/142	22	25	P	5	3.04 P	1.50	4.56 P	+1.5	119/120	27	30	P	5	0.64 P	2.42 P	+1.5
143/144	30	15	P	5	1.92 P		4.16 P	+2.2	33/34	26	26	M	6	3.55 P	5.98 H	+2.4
146/147	23	24	P	6	1.89 P	0.75	2.93 P	+1.1	7/8	21	15	P	6	3.10 P	5.28 P	+2.1
149/150	22	12	P	6	2.76 P	1.25	6.15 P	+3.4	*156/157	26	15	P	8	3.90 H	4.37 P	+0.4
*78/79	1.5	5	P	7	5.44 H	1.50	5.60 P	+0.2	45/47	29	29	M	10	2.24 Dr	5.51 H	+3.3
2																
82/83	1.6	2.5	P	8	4.36 H	2.25	6.30	+2.0	49/51	30	31	M	10	2.92 Dr	6.12 H	+3.2
84/85	2.8	3.3	P	8	3.64 H		9.02	+5.4	160/161	22	40	P	13	4.32 P	5.58 H	+1.2
*88/89	6.3	6.3	P	9	4.64 H	0.75	5.20	+0.6	160/161	22	40	P	13	4.32 P	5.56 H	+1.2
2																
102/103	12	11	P	13	0.00 Dr	0.5	6.45 H	+5.8	*172/173	22	5	M	4	4.96 H	4.36 P	-0.6

All these milks were taken after the 3rd day of lactation. When the fat for these 3 days was examined it was found that in many cases its behaviour was reversed, the fat content being higher in the first sample than in the last. This is shown in Table III. But if the percentages for these milks are carefully scrutinised it appears that this reversal of values occurs only in the case of *primiparae*.

Table III. *Early milk. Fat (%)*.

No.	Sample in cc.		Preg.	Day	Before	Ozs. taken by baby	After	No.	Sample in cc.		Preg.	Day	Before	Ozs. taken by baby	After	
178/179	1.7	1.5	<i>P</i>	1	0.96 <i>H</i>	Nil	0.53 <i>H</i>	71/ 72	1	2	<i>P</i>	2	7.14}	0.25	5.02	
188/189	5	5.5	<i>P</i>	2	3.13 <i>H</i>	0.5	2.72 <i>H</i>	73/ 74	0.7	1.6	<i>P</i>	2	6.92}	1.5	5.74	
181/182	1.7	2.3	<i>P</i>	2	1.66 <i>H</i>	0.25	1.26 <i>H</i>	80/ 81	1.5	5	<i>P</i>	7	9.98	<u>1.5</u>	8.66	
															2	
166/167	4.5	11	<i>M</i>	2	3.50 <i>H</i>	<u>1.25</u>	6.05 <i>H</i>	16/ 17	30	30	<i>M</i>	3	1.57 <i>P</i>	—	2.12 <i>P</i>	
															2	
170/171	3.5	38	<i>M</i>	3	2.97 <i>H</i>	—	4.08 <i>H</i>	168/169	3	11	<i>M</i>	3	3.75	—	4.30	



Now since it is known that the fat in milk is present in the form of an emulsion and is therefore liable to respond, as to its degree of emulsification, to changes in physical conditions, it would appear reasonable that these conditions should play a large part in the determination of the resultant fat percentage in the milk. A consideration of the above figures suggests that the percentage of fat in milk depends upon two factors, the one inversely and the other directly. The first of these is the quantity of liquid available as a suspensory fluid. In a very full breast just before a feed, when stimulation of the one side produces dripping from the other, the fluid will be at a maximum, and therefore, according to this point of view, the fat should be at a minimum. This is exactly what in fact occurs—see cases 102/103, 45/47 and 49/51 (Table II). On the other hand, at the end of a feed when the breast is near exhaustion, the fluid will be at a minimum, and one would expect, therefore, a rise in fat content, and, as will be seen by the above tables, this is in every instance the case. The second factor in operation is the question of direct force applied to the breast itself. This is absent in the case of a milk that drips, at its least in the gentle use of a breast pump, and at its maximum in vigorous digital expression or the action of the jaws of a strongly sucking baby. The influence of this factor is direct upon the proportion of fat in the resulting milk, that is to say, in an opposite direction to the action of the fluid factor. The percentage of fat resulting in the milk depends, therefore, upon a balance of these two forces, the fat rising at the end of a feed if all other conditions are equal. But if greater pressure be applied at the beginning than at the end this rise can be very much modified (78/79, 88/89, 156/157) or even altogether obliterated (172/173).

As a further experiment in confirmation of these contentions, in one case a first sample was taken by vigorous finger expression and set aside. A second sample was then taken by very gentle use of the pump, and finally the remaining milk was withdrawn by hand. The values of the fat found were, first sample 3.88 % (*H*), second 4.08 % (*P*), last 5.34 % (*H*). That is to say, that although the sample with 5.34 % of fat followed immediately upon the sample with 4.08 % and was not very much in excess in quantity, yet the fat difference was 1.26 %. To illustrate both factors at one time, in two other samples a "before" and "after" specimen was taken by pump and hand on the 4th day from the left breast 6 hours after the last feed on that side, and the estimated fat content was 1.62 % and 2.86 % respectively, and the total amount secreted  $3\frac{1}{2}$  ozs. ( $2\frac{1}{2}$  ozs. sucked and 1 oz. expressed). The whole available quantity of the right breast, where the baby had fed 3 hours before, expressed by a pump, reached a total of  $1\frac{3}{4}$  ozs. with a fat content of 2.9 %. That is to say, that on the one side the factor of digital expression plus that of the end of the feed operating in a large quantity of fluid resulted in a fat percentage just equal to that appearing in a very much smaller quantity obtained by the gentler method of expression from the other side.

As the procedure hitherto adopted for the study of the fat content of milk

by most observers has been either to mix the fore milk and strippings [Denis and Talbot, 1919], or to take the middle milk [Adrianse, 1897; Woodward, 1897], it was thought to be of interest to investigate in a series of cases the fat content of these relative to each other. Table IV represents the figures obtained, the second column being the fat percentage in a sample composed of equal parts of first and last milk and the third that of the middle milk of the same sample.

Table IV.

No.	A. + C.	B.	No.	A. + C.	B.
23/23 <i>a</i>	2.36	1.88 ↘	24/24 <i>a</i>	3.70	3.69 →
26/26 <i>a</i>	5.15	5.12 →	27/27 <i>a</i>	4.56	4.78 ↗
28/28 <i>a</i>	4.23	4.51 ↗	29/29 <i>a</i>	3.98	3.63 ↘
38/39	3.34	2.25 ↘	37/37 <i>a</i>	3.65	3.47 ↘
44/44 <i>a</i>	4.19	4.46 ↗	43/43 <i>a</i>	4.26	3.65 ↘

The arrows at the side of the columns indicate by their direction the relative values of the fats of these two columns. It will be seen that the relationship between them is fortuitous.

It is postulated, therefore, that, whereas the other constituents of the milk so far considered vary in percentage according to the general influences of the development of lactation, the percentage of fat depends alone upon factors local to the breast and the manner of extraction of the sample.

*Calcium.* The calcium content of the milk of 18 women was estimated over a period of 2-15 days and according to the same schema as with the other constituents. The following table shows the results obtained in 5 typical cases ranged according to days after parturition.

Table V. *Values for calcium in the milk of 4 women.*

Name	No.	Preg.	Volume of sample cc.	Day	CaO %
McC.	121	P	14	6	0.0342
	122/127	"	5	7	0.0409
	128/129	"	16	8	0.0375
	130/132	"	10	9	0.0457
	133/134	"	15	10	0.0448
L. P.	140	P	57	4	0.0543
	146/151	"	20	6	0.0502
	152/155	"	16	7	0.0502
	158	"	40	12	0.0491
	160/161	"	30	13	0.0463
H. P.	72/ 74	P	2	2	0.0465
	76	"	10	4	0.0498
	77/ 81	"	6	7	0.0466
	82/ 85	"	2.5	8	0.0492
	86/ 89	"	5	9	0.0501
R. J.	90/ 91	"	20	11	0.0465
	15	M	30	2	0.0427
	16/ 17	"	30	3	0.0430
	19/ 22	"	30	4	0.0424
	23/ 28	"	30	5	0.0491
	29	"	30	10	0.0510

NOTE. The figures for calcium given above represent the average figure for all samples estimated on the given day.

It will be seen that no variation is perceptible according to the day of lactation as far as the period 2-15 days is concerned. This naturally does not in any way prejudice the possibility of a later rise and fall with the later development of lactation, as has been suggested by Schabad [1911] and Hunaeus [1909]. It will be seen also that there is a definite regularity in content of the milk of the individual women themselves. This has been the case with all but one of the women investigated, the maximum range of variation among all the samples taken of any one woman being from 0.009 % to 0.017 %. In one case, however, a greater variation occurred, the lowest figure reaching 0.0399 % and the highest 0.628 %, this over a range of 5 days and 20 samples. This can be accounted for either by the hypothesis of Schabad [1911], that such a degree of variation does occur in a certain proportion of women, or by the possible inaccuracies introduced into the work by the use of a breast pump. It would therefore appear that the difference in calcium values shown between the milks of individual women during the period under observation is far greater than that occurring among the samples of milk of any single individual. This fact was also found by Schabad for the milk of the later periods of lactation.

As regards the question of the influence upon the calcium content of the place of the sample in a feed, it has been suggested by Schabad [1911] and Hunaeus [1909] that the value of the calcium falls at the end of a feed. In order to study this point, 22 samples were investigated of the first and last milk of a feed and of that taken before and after a feed. Three specimens were also analysed fractionally from breasts that had dripped. The results are given below:

Table VI. *Variations in single feed. CaO (%)*.

With baby				Without baby			
No.	Before	After	Difference	No.	First	Last	Difference
84/ 85	0.0481	0.0516	+0.003	49/ 51	0.0458 <i>Dr</i>	0.0595 <i>H</i>	+0.014
86/ 87	0.0600	0.0487	-0.011	111/112	0.0369 <i>P</i>	0.0333 <i>P</i>	-0.003
88/ 89	0.0566	0.0571	+0.001	116/117	0.0369 <i>P</i>	0.0369	—
131/132	0.0456 <i>H</i>	0.0453 <i>H</i>	—	119/120	0.0380 <i>P</i>	0.0333 <i>P</i>	-0.005
146/147	0.0476	0.0476	—	160/161	0.0447 <i>P</i>	0.0442 <i>P</i>	—
149/150	0.0521	0.0513	-0.001	40/ 42	0.044 <i>H</i>	0.044 <i>H</i>	—
152/153	0.0477 <i>P</i>	0.0441 <i>P</i>	-0.003	30/ 31	0.0570	0.0626 <i>P</i>	+0.005
16/ 17	0.0410 <i>P</i>	0.0450 <i>P</i>	+0.004	45/ 47	0.0410 <i>Dr</i>	0.0485 <i>H</i>	+0.007
101/103	0.0343 <i>Dr</i>	0.0433 <i>H</i>	+0.009	33/ 34	0.0588 <i>P</i>	0.0485 <i>H</i>	-0.010

If the range of experimental error be taken to be approximately 0.003 % it will be seen that 11 samples show no variation while 5 show a rise and 2 a fall. If, on the other hand, the figures be taken as they stand, 5 are stationary, 7 show a rise and 6 a fall. It would appear therefore, that such differences as occur, are either fortuitous in direction or negligible in amount. We would suggest that, since Schabad gives only 3 samples, and Hunaeus 6, a selection of such as, for instance, 119/120, 86/87 and 33/34 would account for their findings.

When the calcium content of the milk of either breast was compared, the variations found were as follows: in 8 out of 17 cases there was a variation of 0.003 % or under, in 6 out of 17 cases a variation of 0.004 % to 0.008 %, in 3 out of 17 cases variations respectively of 0.014 %, 0.013 % and 0.023 %; for this latter instance no reason is apparent.

In comparing the values for calcium with those for ash, it is worthy of note that the high ash values found at the beginning of lactation are not represented in the calcium. The excess of mineral in the colostrum and early milk must therefore be due to some component other than calcium, possibly magnesium, which, according to Holt, Courtney and Fales [1915], is variable throughout lactation and highest in the colostrum period (considered as 1-12 days) as also are potassium and sodium.

*Solids.* The total solids were estimated in 67 samples from 16 women, and show a variation of from 7.6 % to 18.9 %. But if one case showing 7.6 % and a single example of 18.9 % be omitted, the range of the remaining samples is 9.76 % to 14.98 %. The height of the figures for solids is found consistently throughout all samples to vary in general relationship to the fat and to give an average mean value of 12.68 %.

It is to be noted that the estimated total solids have not, in a considerable number of cases, reached the same total as an arithmetic addition of the results for sugar, fat, protein and ash would give for the same milks, the difference between the two varying from plus 0.046 % to plus 2 %, with a mean of 1.18 %. This is in agreement with the work of Camerer and Söldner [1896] and earlier observers, who found in the case of colostrum and early milk a similar discrepancy. In working with later milk Söldner did not find this discrepancy to occur, and suggested, therefore, that its appearance in the early milk could not be due entirely to experimental error, as had hitherto been suggested, but must represent actual unanalysed substances in the milk. In the analyses recorded in this paper only the precipitable protein nitrogen was estimated. The soluble bodies containing nitrogen therefore, which are known to exist in milk, would necessarily form part of the discrepancy found. Furthermore, the extractives containing no nitrogen which are known to be present in milk, and citric acid, have not been considered.

To sum up, it is suggested that the usual view of milk, as a secretion homogeneous in its response to stimuli, cannot be maintained, and indeed lies at the root of a number of the contradictions and obscurities found in the results of milk analyses. On the contrary, in agreement with the view first put forward by Forster [1881], it is contended that milk is composed of elements opposite in behaviour and very varied in their response to identical stimuli.

## CONCLUSIONS.

Our conclusions with regard to the chemical composition of milk of the first 14 days of lactation and the factors influencing its variation may be summarised as follows.

1. There appears to be a difference between the milk of *primiparae* and *multiparae* with regard to the behaviour, in the early days of lactation, of the protein and fat.

2. The protein and ash content of the milk vary regularly according to the day of lactation, being very high in *primiparae*, less so in *multiparae* on the 2nd and 3rd days, and falling steeply during the first week to reach an approximately normal level of from 0.98 % to 1.3 % for the protein, and 0.20 % to 0.25 % for the ash.

3. The sugar content varies also according to the day of lactation, but inversely as the protein, and less regularly, being at its lowest in the early days and rising irregularly to a normal level of approximately 6.5 % by the end of the second week.

4. The fat content of the milk varies only according to the physical conditions of the breast and the actual extraction, and not in relation to the day after parturition, but in *primiparae* the general behaviour is reversed in the first days of lactation.

5. The calcium content of the milk varies up to the 15th day between individual women, but not according to any factors concerned with individual lactation and is uninfluenced by factors local to the breast.

6. There is some possibility that the protein content of the milk rises slightly at the end of a single feed, the difference being greater when the samples are taken in relation to an actual feed than when only the first and last portions of an artificially emptied gland are considered.

7. The lactose content of the milk appears to be higher at the beginning than at the end of a feed.

8. The total solids vary roughly as the fat, and show a range from 9.6 % to 14.98 %.

We wish to acknowledge our gratitude to Mr Alfred Langton whose generosity, in conjunction with that of the Medical Research Council, has made the research possible. To Prof. Louise McIlroy for putting the material at our disposal and for encouragement in the carrying out of the work, and to Prof. Winifred Cullis for very helpful advice and criticism, we wish to offer our warmest thanks. It is also a pleasure to put on record our appreciation of the able and generous cooperation of Miss Hill, M.B., Third Assistant of the Obstetric Unit, and of the Nursing Staff of the Royal Free Hospital, in the carrying out of the work.

## REFERENCES.

- Adrianne (1897). *Arch. Pediat.* **14**, 22, 85.  
Camerer and Söldner (1896). *Z. Biol.* **33**, 42, 535.  
Carter and Richmond (1898). *Brit. Med. J.* i, 199.  
Denis and Talbot (1919). *J. Dis. Child.* **18**, 93.  
Folin and Denis (1918). *J. Biol. Chem.* **33**, 521.  
Forster (1881). *Ber. deutsch. chem. Ges.* **14**, 591.  
Gardner and Fox (1925). *Practitioner*, **114**, 2, 153, 680.  
Gottlieb (1892). *Landw. Versuch. Stat.* **40**, 1.  
Holt, Courtney and Fales (1915). *J. Dis. Child.* **10**, 229.  
Hunaeus (1909). *Biochem. Z.* **22**, 442.  
McCrudden (1912). *J. Biol. Chem.* **10**, 187.  
Schabad (1911). *Jahrb. Kinderh.* **74**, 514.  
Widdows (1923). *Biochem. J.* **17**, 34.  
Woodward (1897). *J. Exp. Med.* **2**, 217.

## II. A NOTE ON THE SIGNIFICANCE OF GELATIN FOR BACTERIAL GROWTH.

By BENJAMIN STANLEY PLATT (*Beit Memorial Research Fellow*).

*From the School of Medicine, the University of Leeds.*

*(Received November 1st, 1926.)*

IN a study of the effect of various substances present in meat extracts on growth and peroxide formation in pneumococcal cultures it was found that no growth of the strains used occurred in peptone water, and only occasionally in bouillon. The addition of small amounts of Coignet's "Gold Label" gelatin to either of these media always resulted in abundant growth. Previously, Robertson, Sia and Woo [1924] had found that the period of survival of pneumococcus can be considerably increased if 0.1 % gelatin be added to suspensions of these organisms in water or saline. Gelatin purified by Loeb's method also had a beneficial effect on the pneumococcus. The results of the writer's work suggest that 0.1 % commercial gelatin would have some nutritive value for the organism. This is also the view of Meleney and Zau [1924] deduced from the effect of small amounts of gelatin on haemolytic streptococci in saline suspensions.

The growth of pneumococcus in Parke Davis peptone water containing various percentages of gelatin was determined. 3 % gelatin proved to be most suitable. Higher concentrations, up to 16 % gelatin, caused an increase in the duration of the lag period of growth, but viable organisms were present after 3 weeks' incubation. In peptone water containing less than 3 % gelatin, decreases in the amount and in the period of growth were observed.

A 20 % solution of "Gold Label" gelatin, adjusted to  $p_H$  7.8 was autoclaved for periods varying from 20 minutes to 20 hours. No difference in the amount of growth of pneumococcus could be observed in peptone water containing 5 % gelatin from these autoclaved solutions. In the absence of peptone and on the addition of 0.5 % sodium chloride, growth was only obtained in a 15 % gelatin solution autoclaved for 20 hours, and, to a lesser extent, in the sample autoclaved for 18 hours. Growth was also possible in concentrations of 5-10 % of the sample autoclaved for the longest period.

A sample of the commercial gelatin was purified by washing at  $p_H$  4.7 according to the method of Loeb [1922], hydrochloric acid being used instead of acetic acid. All the washings were concentrated *in vacuo*, most of the acid being removed in this process. It was found that growth of the pneumococcus occurred in peptone water containing either the concentrated washings or

the washed gelatin. A mixture of these fractions, in approximately the same proportion as in the original sample of gelatin, had an effect on growth similar to that of the untreated gelatin. Growth was also possible in bouillon on the addition of 2 % purified gelatin.

A portion of the concentrated washings was dialysed in a parchment shell against distilled water at 2°. The concentrated dialysate was found to promote growth of pneumococcus in 2 % washed gelatin-peptone media whilst the dialysed solution had no effect. The ash of "Gold Label" gelatin and of the washings used above have an influence on the growth of the organism similar to that of the dialysate. Furthermore, it was repeatedly found that the addition of 4 % of a Ringer-Locke solution to peptone water allows growth of the pneumococcus to take place in peptone water when washed gelatin is present.

It may be stated in view of the above observations and of other evidence obtained in the course of this investigation that relatively complex protein decomposition products are required for the growth of the pneumococcus. These may be supplied by bacteriological peptones (preferably with a low free amino-acid content) or by hydrolysed gelatin. Secondly, the addition of purified gelatin will allow or will increase growth of pneumococcus in peptone water or in bouillon. Thirdly, in "Gold Label" gelatin there are important impurities which are dialysable and not destroyed by incineration. Ringer-Locke solution may be substituted for these substances.

The following medium was prepared in order to determine whether the recognition of the three foregoing conditions is sufficient to satisfy the growth requirements of the pneumococcus. A 10 % solution of purified gelatin was partially hydrolysed by autoclaving for 24 hours at 120°. Growth of pneumococcus was obtained in a portion of this medium containing the ash of the washings. The media were inoculated with a small amount (0.1 to 2.5 cc. medium) of a dilute suspension of the organism in saline.

Preliminary experiments have been carried out to test the effect of gelatin on the growth of some of the more delicate bacteria other than pneumococcus. Two strains of *B. diphtheriae* were found to grow in a 3 % gelatin-peptone medium and not in either bouillon or peptone water. Better growth of a strain of meningococcus and of two strains of streptococcus was obtained in the gelatin-peptone than in bouillon or peptone water, but the strains of gonococcus examined would not grow in any of these media.

It should be pointed out that the direct assimilation of gelatin by pneumococcus is improbable as this organism does not liquefy gelatin, nor has it, according to Avery and Cullen [1920], any enzyme capable of hydrolysing proteins. Of the many physical properties of gelatin solutions which may affect the growth of bacteria, the decreased solubility and rate of diffusion of oxygen are of particular interest in the case of pneumococcus. Gillespie [1913] has recorded the fact that an inoculum must contain more pneumococci to produce growth in a liquid than on a solid medium. It may be that, before



the initiation of growth of the pneumococcus, a certain minimum value of oxygen tension is essential. This condition, however, is probably realised by the reducing action of the organisms if the area inoculated be sufficiently limited by physical conditions such as obtain in gelatin solutions.

To Prof. J. W. M'Leod, in whose laboratory this work was carried out, I wish to express my thanks for advice and for facilities.

#### REFERENCES.

- Avery and Cullen (1920). *J. Exp. Med.* **32**, 547.  
Gillespie (1913). *J. Exp. Med.* **18**, 584.  
Loeb (1922). *Proteins and the Theory of Colloidal Behaviour*, p. 35. (New York.)  
Meleney and Zau (1924). *J. Exp. Med.* **39**, 811.  
Robertson, Sia and Woo (1924). *J. Exp. Med.* **39**, 199.

### III. PEROXIDE FORMATION BY PNEUMOCOCCUS AND ITS RELATION TO BACTERIAL OXIDATION-REDUCTION REACTIONS.

BY BENJAMIN STANLEY PLATT (*Beit Memorial Research Fellow*).

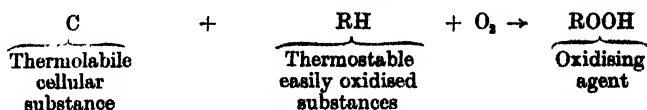
*From the School of Medicine, the University of Leeds.*

*(Received November 1st, 1926.)*

AVERY AND MORGAN [1924] in confirming and extending the findings of M'Leod and Gordon [1922] have summarised the nature of the conditions which must be maintained in order that maximum amounts of peroxide may be produced in cultures of pneumococcus. No mention is made, however, of the effect of the various constituents of the culture media on the amount of peroxide formed, though experiments reported in a later paper [Avery and Neill, 1924, 2] show that peroxide is not formed by washed pneumococci, nor by extracts of washed organisms, unless there is present a substance of undefined character which may be obtained from cell washings, meat or yeast. The organism is usually grown in media which contain substances of animal or vegetable origin, so that, with an adequate supply of oxygen and in the absence of peroxide-destroying agencies, considerable amounts of peroxide have generally been obtained. Pneumococcus does not grow in peptone water, but it has been shown [Platt, 1927] that the addition of a small amount of "Gold Label" gelatin to peptone water provides a medium in which the organism will yield a growth of the same order as that obtained in the presence of meat extract, as, for example, in 3 % gelatin-bouillon. The amount of peroxide produced when pneumococcus is grown aerobically in the former medium is considerably smaller than when the same medium containing meat extract is used. This difference is apparently due to the absence from gelatin-peptone of the substances necessary to complete the peroxide-forming mechanism of the organism. A convenient medium is therefore available for the purpose of determining the nature of these components.

With reference to the function of the "complementary" substances, Avery and Neill [1924, 2] suggest that "the principal rôle may be the furnishing of easily oxidised or autoxidisable substances." In a later publication [Avery and Neill, 1924, 3] they show that the complementary substances are thermostable, and, like the thermolabile component in extracts of washed pneumococci, do not react with molecular oxygen or methylene blue. This opinion has been modified [Neill and Avery, 1925], for it is stated that the thermostable substances react slowly with molecular oxygen forming oxidising agents, and, in anaerobic conditions, methaemoglobin and methylene blue are reduced to

a slight extent. The extract of washed pneumococci is said to catalyse a reaction which is represented by the expression:



Neill and Hastings [1925] and Neill [1925, 2] find that, whilst both oxygen consumption and haemoglobin oxidation by the autoxidisable substances of alcoholic extracts of meat infusion and potato juice are greatly accelerated by the cellular catalyst (C), the reaction with other autoxidisable substances, *e.g.* turpentine, linseed oil and oleic acid, is not affected by the presence of (C). In view of the results of the present research it appears that the substances (RH) required to complete the oxidation-reduction system (C + RH) of the pneumococcus are not mainly the autoxidisable substances of extracts of animal and vegetable tissues of the type found in turpentine. Indeed, were they similar, one would expect that the reactions of both systems would be accelerated alike on the addition of component (C). The suggestion therefore is made that the extracts used, supposed to contain the substances (RH), also contain compounds capable of acting as hydrogen donators. In the presence of the pneumococcus substance (C), hydrogen may be donated to molecular oxygen, which may need to be activated, with the subsequent formation of hydrogen peroxide. Given appropriate conditions, haemoglobin may be oxidised to methaemoglobin and, in the absence of oxygen, methylene blue and methaemoglobin may be reduced. An important hydrogen donator in the case of peroxide-forming bacteria appears to be lactic acid, which may be present in media containing meat or yeast extracts or may be produced by the organism from fermentable substances in the medium.

## EXPERIMENTAL.

### *Methods.*

The estimation<sup>1</sup> of peroxide in the cultures was carried out by adding *M*/100 titanous sulphate in excess, the amount of reagent not oxidised being determined by titration with *M*/100 iron alum solution in the presence of potassium thiocyanate. The conditions specified by Thornton and Chapman [1921] for accurate estimations have been observed. It was found to be more convenient to store the titanous sulphate solution in 4*N* sulphuric acid under hydrogen than to adopt the method advanced by Russell [1926]. All the titrations were carried out in an atmosphere of carbon dioxide. The accuracy of the method for the present purposes was proved by estimating known amounts of peroxide in such culture media as would be employed. Small initial titrations, which remained unchanged on prolonged incubation, were observed in

<sup>1</sup> I am indebted to Mr C. R. Hoskins, B.Sc., of Leeds University, for his assistance with the technique of the peroxide estimations.

sterile media containing meat extract. The titration values for sterile control media are subtracted from those obtained in the following experiments.

The number of viable organisms in a culture was determined by dilution of various amounts with 0.1 % gelatin-saline, several loopfuls of each dilution being plated out on a heated blood-agar medium.

For the preparation of the media Parke-Davis' peptone and Coignet's "Gold Label" gelatin were used. The bouillon was prepared by extracting lean beef with water at 100° for 2 hours (1 l. water to 500 g. beef) and adding 1 % Parke-Davis' peptone and 0.5 % sodium chloride. The media were all adjusted to  $p_{\text{H}}$  7.8 and sterilised by autoclaving. The strain of pneumococcus (pn. 4) was isolated from pus from a case of empyema and was found to be bile-soluble.

*Exp. 1.* 240 cc. of 3 % gelatin-peptone and of 3 % gelatin-bouillon were placed in 3-litre Erlenmeyer flasks, the depth of the medium being about 12 mm. 10 cc. of a 10 hours' culture of a strain of pneumococcus (pn. 4) were used to inoculate each medium. Samples were taken out at intervals, under sterile conditions, for estimations of growth and peroxide. The results obtained are expressed graphically in Fig. 1, where the logarithm of the number of viable organisms per cc. of culture and the amount of peroxide (expressed as cc.  $M/100 \text{ H}_2\text{O}_2$ ) per 10 cc. of culture are plotted against the time of incubation at 37°.

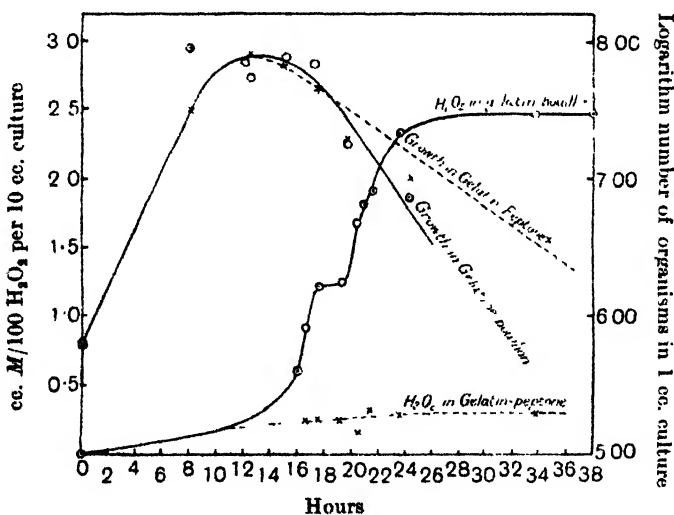


Fig. 1.

It is evident from Fig. 1 that there is a marked difference in the amount of peroxide formed in the two media used, without corresponding differences in the amount of growth. Moreover, peroxide is not produced in gelatin-bouillon in appreciable amounts until near the end of the period of logarithmic increase of the bacteria. Another feature is more clearly demonstrated in the

following experiment when the cultures were incubated at a temperature lower than the optimum for growth.

*Exp. 2.* 10 cc. of 3 % gelatin-bouillon, inoculated with pn. 4 from an 18 hours' culture on heated blood-agar, were pipetted into 250 cc. Erlenmeyer flasks. After incubation at 30° for varying periods, 0.5 cc. was used for a growth estimation, the remainder being titrated with *M*/100 titanous sulphate and *M*/100 iron alum solutions. The results are given in Fig. 2.

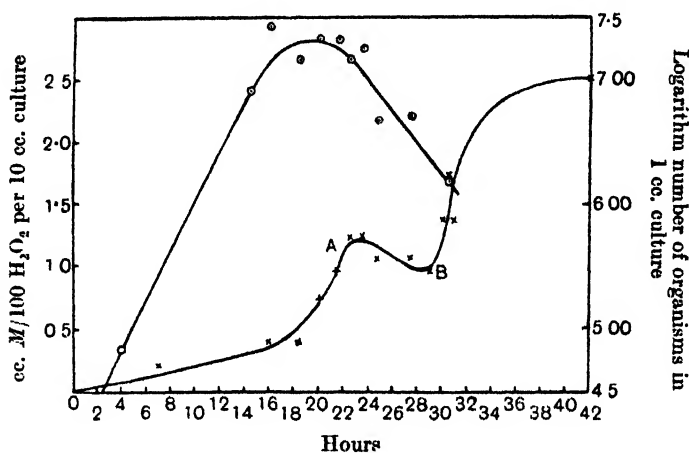


Fig. 2.

The portion (A-B) of the time-peroxide curve given in Fig. 2 appears to be related to the period of stationary growth of the organism and may be due to the effect of the gelatin on the regulation of the oxygen supply to the organism. The organisms which develop before and at the stationary period will exhaust the supply of oxygen, so that there is insufficient for the formation of more peroxide, and there is also a tendency to reduction of that already formed. The latter tendency ceases with the active growth of the organism, whilst the respiration of the autolysed or autolysing bacteria proceeds until the maximum value is attained. That peroxide may be formed in cultures under conditions incompatible with active growth has been demonstrated by Avery and Neill [1924, 1]. Also, inhibitory amounts of peroxide are found in gelatin-bouillon cultures before the maximum value (about 0.01 %) is attained. (Morgan and Avery [1924] show that growth of pneumococcus may be inhibited for 24 hours in a concentration of peroxide of 0.006 %.) There is evidence that more peroxide is formed by the organism than that represented by the actual titration values.

*Exp. 3.* 150 cc. each of 5 % serum-bouillon and 5 % serum-3 % gelatin-bouillon, inoculated from a serum-bouillon culture of pn. 4, were incubated at 37° in 1-litre Erlenmeyer flasks. The ox-serum, which had been sterilised by filtration, was heated to 60° for 30 minutes before use. The data obtained for the rate of growth and peroxide formation are represented in Fig. 3.

From these results it appears that the chemical and physical properties of serum are such as to yield more immediate and regular production of peroxide. The addition of gelatin to serum-bouillon however results in a time-peroxide curve of the type obtained for gelatin-bouillon. It will be seen that the "step" in this curve appears earlier on account of the larger number of organisms produced, as shown in the growth curve.

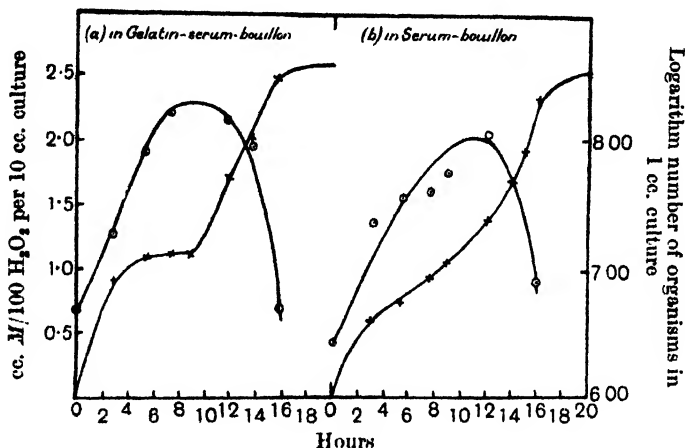


Fig. 3.

*Exp. 4.* In Table I are given the results of some of a number of estimations of peroxide carried out on 10 cc. of 48 hour cultures of pneumococcus in 3 % gelatin-peptone containing sodium lactate. There was no marked difference in the amounts of growth in the various cultures after 12 hours' incubation at 37°.

Table I.

Media	M/100 H <sub>2</sub> O <sub>2</sub>
3 % gelatin-peptone	0.35 cc.
" - 0.02 % sodium lactate	0.78
" + 0.05 % "	1.10
" + 0.1 % "	1.22
" + 1.0 % "	1.10

It is noteworthy that no peroxide is obtained in gelatin-peptone cultures of pneumococcus when a small amount (0.2 %) of sodium thiolactate is present, even when positive tests for peroxide are obtained in control cultures. This substance did not appear to affect the amount of growth.

In a 48 hours' culture of the pneumococcus, containing initially 1 % glucose, a titration of 0.95 cc. M/100 H<sub>2</sub>O<sub>2</sub> was obtained. It will be observed that the peroxide formed on the addition of sodium lactate or glucose to gelatin-peptone cultures is not more than 50 % of the amount usually found in gelatin-bouillon cultures. Small increases in peroxide are found if 1 % glycogen or 0.02 % inositol is present in gelatin-peptone cultures. The meat extract no doubt furnishes other substances from which, directly or in the

course of metabolism, peroxide is produced. Quastel [1926] finds that the effect of mixtures of hydrogen donors is equal to the sum of the activities of the separate constituents. The probability is that a similar relationship exists for the constituents of a medium taking part in peroxide formation.

#### DISCUSSION.

In addition to the foregoing observations, there are other lines of evidence which point to the important part played by lactic acid in bacterial oxidation-reduction reactions [cf. McLeod, Gordon and Pyrah, 1923]. A comparison of the curves in the above figures, which are typical of a number obtained, with that given by Morgan [1924] for the rate of change in  $p_H$  of a culture of pneumococcus also suggests that the peroxide and acid production are related. Further, it has been observed that aerobic cultures, in which there is active peroxide formation, invariably develop a more acid reaction than those in which only small amounts of peroxide are produced. This difference is not determined by the amount of growth. Valentine [1926] has recorded interesting variations in the properties of two strains of streptococci X and Y. Strain X produces much less peroxide and less acid than strain Y. The latter strain has also a much wider range of fermentation reactions.

There is undoubtedly a close parallelism between the reducing and peroxide-forming activities of extracts of pneumococcus [Avery and Neill, 1924, 3], so that it may reasonably be assumed that under appropriate conditions lactic acid will complete the reducing as well as the peroxide-forming systems. A comparable mechanism appears to exist in washed *B. coli* which, in the presence of lactic acid, has a marked reducing action [Quastel and Whetham, 1925, 1]. No peroxide formation however will be evident, on account of the presence of catalase in *B. coli*. It is significant that yeast reacts towards lactic acid in a similar manner and that both yeast and *B. coli* appear to metabolise lactic acid only when an ample supply of oxygen is available.

Glucose has been found to promote peroxide formation in gelatin-peptone cultures of pneumococcus. Now Neill [1925, 1] has shown that glucose does not activate extracts of washed pneumococci, but only completes the oxidation-reduction system of the intact cell. It therefore appears to be necessary that, before glucose can function as a hydrogen donor, fission of the glucose molecule must occur, probably with the formation of lactic acid. Quastel and Whetham [1925, 2] find that glucose has a high "reducing coefficient" in the presence of resting *B. coli*. Whether in this instance the glucose undergoes metabolism cannot be determined from available data.

In conclusion, it is suggested that, taking into account the relation of lactic acid to the type of bacterial reaction under discussion, less lactic acid would be formed in aerobic cultures of lactic acid-forming bacteria than in anaerobic cultures. This may explain the recent results of Rona and Nicolai [1926] and of Neuberg and Gorr [1926].

## SUMMARY.

1. Cultures of pneumococcus have been obtained, which, though aerated and free from catalase, yet produce only small amounts of peroxide. A considerable amount of peroxide is formed when cultures contain meat extract. This difference in behaviour is not determined by the amount of growth in the two media.

2. The rates of growth of, and peroxide formation by, pneumococci are compared under various conditions. The effect of oxygen supply on the rate of peroxide formation is discussed.

3. An increase in the amount of peroxide formed is observed in pneumococcal cultures containing lactic acid or glucose. It is suggested that the lactic acid functions as a hydrogen donator in the presence of the pneumococcus, peroxide being formed when oxygen acts as hydrogen acceptor.

4. The influence of glucose and of lactic acid on some reactions of pneumococci is discussed and correlated with observations on other organisms.

## REFERENCES.

- Avery and Morgan (1924). *J. Exp. Med.* **39**, 275.  
Avery and Neill (1924, 1). *J. Exp. Med.* **39**, 347.  
——— (1924, 2). *J. Exp. Med.* **39**, 357.  
——— (1924, 3). *J. Exp. Med.* **39**, 543.  
M'Leod and Gordon (1922). *Biochem. J.* **16**, 499.  
M'Leod, Gordon and Pyrah (1923). *J. Path. Bact.* **26**, 127.  
Morgan (1924). *J. Exp. Med.* **39**, 565.  
Morgan and Avery (1924). *J. Exp. Med.* **39**, 335.  
Neill (1925, 1). *J. Exp. Med.* **41**, 299.  
——— (1925, 2). *J. Exp. Med.* **41**, 551.  
Neill and Avery (1925). *J. Exp. Med.* **41**, 285.  
Neill and Hastings (1925). *J. Biol. Chem.* **63**, 479.  
Neuberg and Gorr (1926). *Biochem. Z.* **173**, 476.  
Platt (1927). *Biochem. J.* **21**, 16.  
Quastel (1926). *Biochem. J.* **20**, 166.  
Quastel and Whetham (1925, 1). *Biochem. J.* **19**, 520.  
——— (1925, 2). *Biochem. J.* **19**, 645.  
Rona and Nicolai (1926). *Biochem. Z.* **172**, 82.  
Russell (1926). *J. Chem. Soc.* **129**, 497.  
Thornton and Chapman (1921). *J. Amer. Chem. Soc.* **43**, 91.  
Valentine (1926). *J. Infect. Dis.* **39**, 29.



## IV. A NOTE ON PLANT OXIDATION: THE NATURE AND REACTIONS OF THE SUBSTANCE "TYRIN."

BY BENJAMIN STANLEY PLATT AND ARTHUR WORMALL.

*From the Department of Physiology, the School of Medicine, Leeds.*

*(Received December 31st, 1926.)*

IN an investigation of the oxidising systems of the potato, Szent-Györgyi [1925] obtained a preparation which, he states, plays an important rôle in this plant oxidation. This preparation, to which he gave the name "tyrin," is capable of "oxidation" to a red compound ("oxytyrin") by *ortho*-quinone or by the system potato oxidase-catechol. Szent-Györgyi represents the changes occurring in the potato as follows:

Mol.  $O_2$  + oxidase + catechol  $\rightarrow$  *o*-diketoquinone.

*o*-Quinone + leuco-tyrin (reduced form)  $\rightarrow$  catechol + oxytyrin (red compound).

Oxytyrin + "active" hydrogen  $\rightarrow$  leuco-tyrin.

According to Szent-Györgyi, "tyrin" is concerned in the oxidations of the potato and possibly other plant and animal tissues, as similar preparations could be obtained from various mammalian and other tissues, and he concludes that "tyrin" can be classified as a member of Palladin's group of respiratory pigments.

Happold and Raper [1925] have shown that tyrosinase will act upon phenolic compounds in the presence of amino-acids to give deeply pigmented substances only when the phenol is likely to furnish an *ortho*-quinone on oxidation. In these cases, pigment formation is accompanied by deamination. *o*-Benzoquinone alone produced the same results as the system tyrosinase-catechol (phenol or *p*-cresol). On these grounds a satisfactory explanation was afforded of the liberation of ammonia and pigment formation in the Chodat *p*-cresol-tyrosinase and similar reactions.

In the light of the experiments of Happold and Raper it appeared possible that the properties and reactions of "tyrin" were partly or wholly due to amino-acids, peptides or compounds of these with carbohydrates [cf. Borsook and Wasteneys, 1925]. Preparations of "tyrin" made by Szent-Györgyi's method have all contained amino-nitrogen in appreciable quantity and it has been found that the "oxidation" reactions of these preparations can be accounted for by the presence of nitrogenous compounds. The reactions of "tyrin"—the formation of a red compound on treatment with *o*-quinone or the catechol-oxidase system, the reduction of this compound with NaHS

etc.—can all be produced to approximately the same extent with solutions of various amino-acids or peptone containing the same amount of nitrogen. Thus “tyrin” appears to be a mixture of nitrogenous compounds, especially amino-acids, and to have no other significance in oxidation processes.

#### PREPARATION AND PROPERTIES OF “TYRIN.”

The method of preparation was that described by Szent-Györgyi [1925], the first product being a thick oily substance, from which, however, it has been found possible to prepare a white stable powder by precipitation from a methyl alcohol solution with ethyl alcohol. The powder obtained had all the properties of the original sticky mass. Szent-Györgyi records a negative Millon’s test for tyrosine, but a small amount of this amino-acid has been detected in all preparations, when care has been taken to avoid excess of the reagent. The presence of a trace of tyrosine has been confirmed by the Folin and Denis colour reaction [1912].

<i>Analysis.</i> Total nitrogen (micro-Kjeldahl)	5.7 %	3.6 %
Free amino-nitrogen (formaldehyde titration)	1.4 %	1.3 %

#### *Relationship between “tyrin” and amino-acids.*

If the pigmentation of “tyrin” under the influence of *o*-quinone or the catechol-oxidase system is due to the amino-acids present, liberation of ammonia should occur as in the experiments of Happold and Raper [1925]. A definite increase in the ammonia content of the solution during this pigmentation was observed, the determinations being carried out wherever possible by two methods, the aeration method (after addition of potassium carbonate), and the permuted filtration method of Whitehorn [1923]. Experimental details have been described previously [Happold and Raper, 1925; Raper and Wormall, 1925].

Solutions of glycine, alanine and glutaminic acid containing the same amount of nitrogen as a solution of “tyrin” were prepared and the properties of all solutions compared in the following respects.

(a) Red pigment formation with *o*-quinone—this pigmentation was approximately equal in the case of “tyrin” and glycine and somewhat less in the other amino-acids.

(b) Red pigment formation in the presence of catechol and potato oxidase at  $p_H$  6.4—similar results to those in (a).

(c) Pigmentation with *o*-quinone after treatment with neutral formaldehyde—“tyrin” and amino-acid solutions were treated with varying amounts of neutral formaldehyde, and the solutions neutralised and tested with *o*-quinone after the addition of buffer solution at  $p_H$  6.4. All solutions still retained the power of reacting with the quinone to form red compounds.

(d) Pigmentation with *o*-quinone after treatment with nitrous acid—“tyrin” solutions and amino-acid solutions behaved identically, the ability to react with *o*-quinone being destroyed almost completely in every case.

From these results the conclusion has been drawn that amino-acids, free and combined, present in the "tyrin" are solely responsible for the red pigment formation with *o*-quinone or with the catechol-oxidase system. Equivalent amounts of some of the more reactive amino-acids or peptone will give pigment formation to the same extent. "Tyrin" appears to contain, among other substances, a mixture of amino-acids, some simple peptides and compounds of these with reducing sugars.

#### THE CATECHOL-OXIDASE SYSTEM. *o*-QUINONE AND PEROXIDE FORMATION.

Szent-Györgyi [1925] produced evidence that the guaiacum reaction obtained with potato oxidase in the presence of catechol is due to the formation from the catechol of *o*-quinone and that the enzymes present take no further part in the blueing of guaiacum. These conclusions are based on the fact that if potato oxidase is allowed to act on catechol for 10 minutes at 37° and the enzymes precipitated with methyl alcohol, the enzyme-free filtrate will blue guaiacum. *o*-Quinone prepared by the method of Willstätter and Müller [1908] gave the same blueing of guaiacum, was inactivated by excess of catechol and showed the same general lability as the substance formed in the catechol-oxidase reaction. Proof of the identity of the reactive substance formed in the enzyme reaction was not furnished, but, recently, evidence of the formation of *o*-quinones from phenolic compounds under the action of the tyrosinase oxidase has been published [Happold and Raper, 1925; Raper, 1926]. Szent-Györgyi therefore divides the blueing of guaiacum by the catechol-oxidase system into two phases:

- (a) catechol + oxidase + oxygen  $\rightarrow$  *o*-quinone,
- (b) oxidation of guaiacum by the oxide of catechol (*o*-quinone),

for which no enzyme is required.

The removal of enzymes from the solution was effected by Szent-Györgyi by the addition of methyl alcohol and filtration; but this does not preclude the possibility of small amounts of peroxidase or oxidase being present in the filtrate. These experiments, with additional controls, have therefore been repeated, using a method which is easy to carry out and which does not appear to be open to this objection. Potato oxidase was allowed to act on a catechol solution at  $p_H$  6.4 for 10 minutes, the solution extracted with ether and the ethereal solution tested with guaiacum and benzidine. Positive results were obtained, while all the controls—catechol alone, enzyme alone and catechol + heated enzyme—gave negative results. Since these experiments were carried out, Onslow and Robinson [1926] have also confirmed the observations of Szent-Györgyi, a method involving precipitation of the enzymes with "dialysed iron" being used. As these authors admit, however, the use of "dialysed iron" in connection with guaiacum is open to objection, although their control tests gave negative results.

*Experimental procedure.* The enzyme used in these experiments was prepared by the method used by Szent-Györgyi [1925] which is based on that of Onslow [1920].

The following solutions *A*, *B*, *C* and *D* were prepared:

	0.1 % catechol cc.	Phosphate buffer ( $p_H$ 6.4) cc.	Distilled water cc.	Potato oxidase mg.
<i>A</i>	2	5	3	100
<i>B</i>	—	5	5	100
<i>C</i>	2	5	3	—
<i>D</i>	2	5	3	100 (heated)

The solutions were incubated at 37° for 5 minutes, each solution was shaken thoroughly with 15 cc. pure ether, and the ethereal extracts were pipetted off and filtered. These ether extracts were tested in the following manner with an alcoholic solution of guaiaconic acid and 1 % alcoholic benzidine.

	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>
3 cc. ether extract + 3 drops alcoholic benzidine	Blue	—	—	—
3 cc. ether extract + 3 drops alcoholic guaiaconic acid + 2 cc. distilled water	„	—	—	—

These experiments have been repeated a large number of times and in no case did the controls give any colour. Specially purified ether has been used for the extractions, but this does not appear to be essential, for negative controls are obtained with once distilled ether. The blueing of guaiacum and benzidine in ethereal solutions appears to preclude absolutely the possibility of enzyme interference in this phase of the reaction.

In the course of an investigation of the production of peroxides from catechol and other phenols, solutions containing catechol and potato oxidase have been examined for hydrogen peroxide, with positive results. Onslow and Robinson [1926] have also demonstrated the production of hydrogen peroxide in such solutions and in solutions of catechol which had been allowed to oxidise by exposure to the air for several days.

This formation of hydrogen peroxide in the catechol-oxidase reaction, although slight, for most of that formed will quickly be decomposed by the catalase present or utilised by the peroxidase for oxidation purposes, can readily be demonstrated by the following method, which does not necessarily involve the use of ether. This solvent usually contains a peroxide which will give the titanium sulphate test for hydrogen peroxide and therefore has to be specially purified if used in connection with these tests.

*Experimental procedure.*

	1.0 % catechol drops	Phosphate buffer ( $p_H$ 6.4) cc.	Distilled water drops	Potato oxidase mg.
<i>A</i>	5	5	—	50
<i>B</i>	5	5	—	—
<i>C</i>	5	5	—	50 (heated)
<i>D</i>	—	5	5	50

These solutions were dialysed in parchment sacs against 20 cc. distilled water in each case. Samples (2 cc.) of each dialysate were withdrawn after 5, 10 and 30 minutes and tested for hydrogen peroxide by the addition of 0.5 cc. of 25 %  $\text{H}_2\text{SO}_4$  and varying amounts of titanium sulphate in 5 %  $\text{H}_2\text{SO}_4$ . The presence of peroxide, presumably  $\text{H}_2\text{O}_2$ , in the dialysate from solution A only was indicated by a slight yellow coloration. A stronger reaction with the titanium sulphate reagent was obtained by carrying out the reactions on a larger scale, acidifying the dialysate with  $\text{H}_2\text{SO}_4$  and concentrating under reduced pressure. The concentrated dialysates gave a yellow coloration only in the case of solution A. The slight yellow colour obtained with some of these controls was apparently due to the catechol, since no colour was produced if the solutions had been extracted previously with purified ether, as suggested by Onslow and Robinson [1926]. This treatment, however, in no way affected the positive peroxide tests with solution A.

#### CONCLUSIONS.

1. The "tyrin" preparation of Szent-Györgyi has been shown to contain appreciable amounts of amino-acids.
2. All the oxidative properties attributed to this preparation can be explained by the presence in it of free or combined amino-acids and can be imitated exactly by a mixture of amino-acids. "Tyrin" therefore plays no significant rôle as a respiratory pigment.
3. The suggestion made by Szent-Györgyi as to the mechanism of the blueing of guaiacum by the catechol-oxidase system has been confirmed by a method which precludes the possibility of enzymic action in the second part of the reaction. The production of hydrogen peroxide in addition to this oxidation product (presumably *o*-quinone), which blues guaiacum directly, has also been demonstrated.

#### REFERENCES.

- Borsook and Wasteneys (1925). *Biochem. J.* **19**, 1128.  
Folin and Denis (1912). *J. Biol. Chem.* **12**, 245.  
Happold and Raper (1925). *Biochem. J.* **19**, 92.  
Onslow (1920). *Biochem. J.* **14**, 535.  
Onslow and Robinson (1926). *Biochem. J.* **20**, 1138.  
Raper (1926). *Biochem. J.* **20**, 735.  
Raper and Wormald (1925). *Biochem. J.* **19**, 84.  
Szent-Györgyi (1925). *Biochem. Z.* **162**, 399.  
Whitehorn (1923). *J. Biol. Chem.* **56**, 751.  
Willstätter and Müller (1908). *Ber. deutsch. chem. Ges.* **41**, 2580.

## V. THE MICRO-ESTIMATION OF CHLORINE IN WHOLE BLOOD SERUM OR CORPUSCLES.

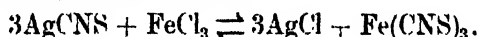
BY FREDERICK HORACE SMIRK.

*From the Laboratory for Clinical Research, Manchester Royal Infirmary.*

*(Received November 15th, 1926.)*

### PREVIOUS METHODS.

CLAUDIUS [1924] described a method of micro-estimation of blood chloride in which he treated 0.2 cc. of blood with 20 cc. of *N*/25 silver nitrate and a few drops of nitric acid. After boiling this down to 0.25 cc. he completed the oxidation with potassium permanganate. Alcohol was added and the solution titrated with alcoholic ammonium thiocyanate. The use of alcohol was a considerable advance, but is open to the objection that it forms an explosive mixture with high concentrations of nitric acid. When titration is carried out in the presence of silver chloride, a large excess of nitric acid is necessary owing to the reversibility of the reaction



In addition, boiling down to small bulk is tedious and frequently causes loss by bumping.

Rehberg [1925] recently overcame these difficulties by maintaining a large excess of nitric acid, but keeping the reacting materials in small bulk. He utilised the special method of titration described by himself. The blood was digested with 100 volume hydrogen peroxide as introduced by Fowweather [1926] for blood-iron estimations. A single estimation of whole blood is completed in 2 hours, for the most part occupied by digestion in a water-bath.

The following method, using ammonium persulphate and nitric acid on 0.2 cc. of blood, gives a smaller margin of error for whole blood than Rehberg's method; it can be completed in 15 minutes for a single sample, or 12 minutes per sample over a series of estimations. It has the advantage that acetone intensifies the end-point more than alcohol and can be used in the presence of high concentrations of nitric acid.

### PRINCIPLE OF THE METHOD.

The principle of this method is the destruction of the organic matter of the blood by ammonium persulphate and concentrated nitric acid, with the usual precipitation of the chlorine as silver chloride by excess of silver nitrate. The excess is titrated against alcoholic ammonium thiocyanate solution with concentrated ferric iron alum as indicator.

The titration is performed in the presence of acetone which intensifies the end-point by decreasing the dissociation of the ferric thiocyanate, and for a similar reason renders it more permanent. This enables a series of titrations to be brought to the same depth of red, which with the sharpened gradation of the end-point coloration permits accurate comparative observations to be made.

#### PROCEDURE.

Deliver 0.2 cc. of blood from a standardised capillary pipette to the bottom of a Jena or Pyrex test-tube. Wash the pipette carefully with 3 or 4 drops of distilled water contained in a small centrifuge tube and transfer the washings by means of the pipette to the Jena tube; *repeat* the washings twice. Add 0.5 cc. of aqueous silver nitrate (7.265 g. per litre; 1 cc. = 0.0025 g. NaCl), using a standardised Ostwald pipette. After mixing these, add very approximately 0.33 g. of pure powdered ammonium persulphate and wash down the adhering particles of persulphate and added liquids with 3.0 cc. of concentrated nitric acid (chlorine-free) from a pipette or a burette with ungreased tap.

Heat this mixture gently with moderate agitation and avoidance of frothing till the protein has dissolved. Clamp the test-tube in a retort stand at about 60° to the horizontal and bubble air through its contents by a fine capillary tube not necessarily of Jena glass (see Fig. 1).

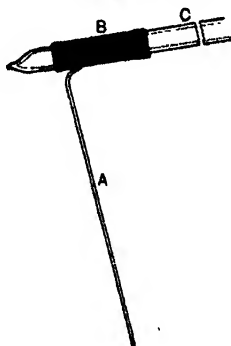


Fig. 1. A. Capillary tube.  
B. Short rubber tube carrying the capillary tube A in a slit.  
C. Glass tube to air supply.

Bumping is entirely prevented by the stream of air bubbles, and the liquid is boiled freely for 15 or 20 seconds, using a small Bunsen burner or a larger size of microburner. The opalescence of the silver chloride precipitate is replaced by a clear greenish yellow solution containing small compact clumps or granules of silver chloride. At this stage the flame is removed and the capillary tube detached from the valvular slit in the rubber tube delivering air. The test-tube, carrying with it the capillary tube, is removed from the stand and allowed to cool.

All the samples of a series are brought to this stage and cooled thoroughly; after which air is again bubbled through by means of the capillary tube which is very readily attached and detached. 3.5 cc. of acetone are added to each

in turn from a small measuring cylinder, the air bubbles ensuring complete mixing. When more than 6 samples are in progress they should be treated with acetone and carried to the final stage in two groups; but if no more than 6, all may go through together.

When the acetone has been added to the last sample the heat of mixing will have passed off sufficiently in the first of the series, and, again connecting the air supply to its capillary tube, 0.3 cc. of concentrated ferric iron alum solution is added. A whitish precipitate usually forms due to the insolubility of the alum in acetone, and distilled water should be added drop by drop till it dissolves (usually 6 drops).

The Jena tubes are best ranged in an ordinary single row test-tube stand, with a white porcelain plate behind them and white paper beneath, using a strong front illumination from a shaded electric lamp about 6 inches away.

The first sample is then titrated against an alcoholic solution of ammonium thiocyanate from a 5 cc. microburette delivered directly into the solution by a fine capillary extension of the microburette attached by pressure tubing. The bubbles of air produce effective stirring, and the end-point is very sharp and sensitive in this illumination, especially in a darkened room. The observer's eyes should be completely protected from glare by a shade on the lamp. Titration is carried in the first sample to the earliest definite pink, and subsequent samples should be titrated by comparison to the depth of colour of the first sample.

Having completed the whole series, the samples are then transferred to tubes of colourless glass and of exactly equal bore. The solutions are carefully poured out, leaving the last few drops containing the silver chloride in the Jena tubes, and titration is then carried accurately to the colour of the first sample. Deeper solutions, if any, are corrected by titrating a solution of the same depth as the first sample to the colour of the darker solution, and subsequently subtracting the excess added from the titre of that solution. Corrections usually amount to about 0.02 cc. of alcoholic thiocyanate.

Although there are several operations, it is found in practice that they are much easier to perform than to describe and there is very little to go wrong.

*Certain supplementary points in technique, which must be carefully followed.*

(1) Pipettes used must be carefully re-standardised; the 0.2 cc. with mercury, as their contents are to be washed out, and the 0.5 cc. Ostwald pipettes with water as they are to be blown out after 15 seconds' drainage. The Ostwald pipettes should deliver their contents slowly, about 30 seconds as a minimum time, or wetting of the glass causes appreciable error. Iron alum is added grossly in excess of that required to combine with the thiocyanate.

(2) Dissociation is also less in acetone; therefore titration is performed, in the presence of acetone, with the minimum of water for solution of the iron alum. The standard ammonium thiocyanate cannot therefore be used in acetone solution because the alum would be precipitated by the acetone added



in the course of the titration. Dissociation is much less in alcohol than in water, though greater in alcohol than in acetone. Therefore alcoholic ammonium thiocyanate is the best practicable substitute for a solution in acetone.

(3) Ferric thiocyanate dissociates into ferric and thiocyanate ions which are colourless. The minimum dissociation takes place when there is a marked excess of either thiocyanate or iron. Therefore, to obtain the maximum coloration with the minimum of thiocyanate excess, a large quantity of iron must be added.

(4) Very occasionally a sample turns pink 0.1 or 0.2 cc. before the true end-point—especially if the thiocyanate standard has been run in too quickly. These samples always fade completely before the final accurate colour comparison is made and no gross error results when the final correction is added.

(5) Brownish colours in the mixture before titration are due to incomplete cooling before or after addition of acetone. If samples are allowed to stand this colour disappears in half-an-hour.

*Results of 9 consecutive observations on whole blood.*

Total chloride per 100 cc.	Known chloride added per 100 cc.	Chloride of original blood sample
g.	g.	g.
0.429	0.000	0.429*
0.446	0.025	0.421
0.424	0.000	0.424
0.468	0.044	0.424
0.519	0.094	0.425
0.423	0.000	0.423
0.452	0.031	0.421
0.423	0.000	0.423
0.517	0.094	0.423

*Results of 9 consecutive observations on serum.*

Total chloride per 100 cc.	Known chloride added per 100 cc.	Chloride of original serum sample
g.	g.	g.
0.553	0.000	0.553
0.581	0.025	0.556
0.553	0.000	0.553
0.602	0.044	0.558
0.557	0.000	0.557
0.653	0.094	0.559
0.558	0.000	0.558
0.574	0.025	0.549*
0.596	0.038	0.558

\* No known cause for this deviation. Rehberg claims errors of only 2 or 3 mg. in his estimations of serum chloride, using 0.1 cc. of serum. This amounts to an error of  $\frac{1}{3}$  or  $\frac{1}{4}$  %. Such results have frequently been obtained on a short series of estimations. Errors in the second figure are very rare. The published series contain the error due to pipette calibration since a different pipette was used for each sample. A closer approximation of results can be obtained by using the same pipette throughout.

## SUMMARY.

- (1) A simple and rapid method of estimating blood chlorides on 0.2 cc. has been described.
- (2) Using whole blood the outside error is usually 4 milligrams of NaCl per 100 cc. of blood, with a similar percentage error for serum.
- (3) Larger errors occur for unknown reasons in about one-tenth of the samples, but remain in the third place of decimals.

I wish to thank Prof. Raper and Prof. Craven Moore for their interest in this work, and Dr Roberts for testing the accuracy of the method.

## REFERENCES.

- Claudius (1924). *Acta Medica Scand.* 61, 3.  
Fowweather (1926). *Biochem. J.* 20, 93.  
Rehberg (1925). *Biochem. J.* 19, 270.

[Note added February 4th, 1927]. Although the method of titration already described, followed by correction in comparator tubes, is the better for occasional use and for those workers whose colour sense is not acute, a modification has been introduced since going to press, which yields better results in the hands of practised workers whose colour perception is good. The acetone is added to the cooled digested samples, taking not more than two or three of a series at once. The mixture is then cooled for about 30 seconds and titrated directly with ammonium thiocyanate. The thiocyanate is run in at a moderate rate and a *brisk* stream of air bubbles is passed to ensure *rapid* mixing. Titration is complete at the first distinct uniform red flush which appears throughout the solution.

Results in duplicate by this method compare very closely. The error is usually about 1 to 3 milligrams, occasionally 6 or 8 milligrams, in the serum and whole blood and about 6 or 8 milligrams in the corpuscles. Larger errors than 8 milligrams are rare in the corpuscles provided that the corpuscular mass has been thoroughly mixed.

The time of estimation is reduced by this modification to an average of 9 or 10 minutes per sample over a series, and 13 minutes for a single estimation.

## VI. THE MICRO-ESTIMATION OF IRON IN BLOOD.

By FREDERICK HORACE SMIRK.

*From the Laboratory for Clinical Research, Manchester  
Royal Infirmary.*

*(Received November 15th, 1926.)*

THE estimation of iron in small quantities of blood is almost universally colorimetric. The thiocyanate colour is preferable to the ferrocyanide because of its greater intensity and the unsuitability of blue solutions for colorimetric work.

The use of acetone to intensify colour and minimise the influence of interfering substances was introduced by Mariott and Wolf [1906] and used by Fowweather [1926]. It was not used by Walker [1925] and was criticised by Wong [1923] who considered that its evaporation introduced considerable errors. Personal observations strongly confirm Fowweather's contention that this error is negligible and entirely outweighed by the advantages outlined by Mariott and Wolf.

Destruction of organic matter has been accomplished by concentrated hydrobromic acid [Berman, 1918] and by potassium chlorate [Wong, 1923], and recently Fowweather has published another method using Wong's procedure, but replacing the potassium chlorate by perhydrol (100 volume  $\text{H}_2\text{O}_2$ ) in order to prevent the violent bumping and frequent loss of samples with potassium chlorate.

Using Fowweather's method occasional bumping and loss of samples was still troublesome. With different samples of perhydrol, oxidation was found to be somewhat tedious. It appeared that his order of accuracy would have been attained with practice. The following has been elaborated as a shorter method for a series of estimations, entirely without risk of bumping and with probably a greater accuracy than that claimed by Fowweather.

The method depends on the rapid oxidation of blood proteins by ammonium persulphate and nitric acid, with subsequent colorimetric estimation of iron as thiocyanate in the presence of acetone, against an artificial colour standard. This enables a series of estimations to be made using the same standard whereas alternatively for accurate working it becomes necessary to make an iron standard for each sample.

## PROCEDURE.

0.2 cc. of blood is delivered from a standardised pipette to the bottom of a  $4\frac{1}{2}$  or 5 inch Jena or Pyrex test-tube. The pipette is washed by sucking two or three drops of water up and down the pipette from a small centrifuge tube and then transferring these by the pipette to the Jena tube. The process of washing is repeated twice.

0.33 g. of a well mixed sample of powdered ammonium persulphate is added to the contents and washed down to the bottom of the tube with 2 cc. of concentrated nitric acid.

The mixture is gently heated with moderate shaking till the protein is dissolved and the solution is clear. The tube is clamped in a retort stand and heating is continued for about 1 minute, air being bubbled through the solution from a fine capillary tube in order to prevent bumping. The solution darkens slightly in shade and then grows lighter again. This may be taken as an index of complete oxidation though the slight colour change is not always noted. The flame is withdrawn and 6 cc. of distilled water are added from a burette, the stream of air ensuring mixing. The capillary tube is then withdrawn and inserted without washing into the next of the series of samples.

The first sample, which has now been boiled and diluted with 6 cc. of water, is poured into a 50 cc. flask and the test-tube washed out three times with 2 cc. of distilled water from a burette, each time adding the washings to the 50 cc. flask. The test-tube is then placed on clean blotting paper by the flask ready for the final washing with acetone.

When all the samples are dissolved and delivered into 50 cc. flasks, 25 cc. of acetone are taken in a small measuring cylinder and the first of the series of test-tubes which has already been washed with distilled water is washed out again with the acetone, the whole 25 cc. of acetone washings being delivered to the first flask which is then gently shaken and immersed in cold water for 5 minutes. 5 cc. of concentrated ammonium thiocyanate solution (24 g. per 100 cc. of water) are added and the volume is made up to the 50 cc. mark with distilled water. The iron estimation is then made colorimetrically against an artificial standard, consisting of a mixture of cochineal, methyl red, and hydrochloric acid dissolved in an equal mixture of acetone and water. The proportions are as follows:

4 cc. cochineal solution (British Drug Houses' Indicator).

3.2 cc. methyl red solution (British Drug Houses' Indicator).

4 cc. dilute hydrochloric acid (1 in 5).

This is diluted with equal parts of acetone and water to the approximate depth of colour required and a final adjustment of tint is made by adding drops of methyl red if the orange tint predominates, and of cochineal solution if the red is too clear. The colour is permanent and indistinguishable from that of the thiocyanate.

Before the ammonium thiocyanate is added to the first of a series of estimations it is best to add the acetone washings to the second of the series so that this may be cooling while the colorimetric readings are taken on the first sample.

Finally 1 cc. of a solution containing 0.1 g. of ferric iron per litre (*v. Flow-weather*) is added from an Ostwald pipette to a Jena tube. 0.33 g. of ammonium persulphate and 2 cc. of concentrated nitric acid are also introduced and these are boiled down to about 2 cc. as are the samples containing blood. The solution is made up to 50 cc. as already described and compared with the artificial standard. A blood sample of equal tint to this iron sample contains 50 mg. of iron per 100 cc. of blood.

In explanation it is well to point out that ammonium persulphate as supplied by manufacturers contains minute traces of iron. Hence a well-mixed sample must be used and the error balanced by a similar addition to the iron standard. Iron-free nitric acid also gives a slight coloration with thiocyanate which is corrected by the nitric acid in the standard.

#### PRACTICAL PRECAUTIONS.

(1) Slight differences in the opacity of solutions always cause trouble in colorimetric work. These can be corrected by increasing the illumination on the opaque side.

(2) Bubbles of gas are set free when acetone is mixed with water. The solutions should be shaken and these allowed to escape or there will be great difficulty with the colorimeter.

(3) The colorimeter is the main source of error. When the colorimetric readings are close and the results divergent the probability is that the illumination has been unequal. The illumination is best adjusted between each reading. 15 mm. is the best level for most people when the iron content is normal, and 20 mm. for anaemic bloods. The personal factor largely governs other optimal conditions for any one observer.

#### RESULTS.

*Results of 18 consecutive observations, made with two different samples of human blood A and B.*

<i>Sample A of blood</i>	Total estimated iron in sample mg.	Known added iron mg.	Total iron minus added iron = iron in 0.2 cc. of blood taken mg.
1	0.109	0.004	0.105
2	0.106	0.000	0.106
3*	0.108	0.006	0.102
4	0.110	0.003	0.107
5	0.107	0.000	0.107
6	0.112	0.005	0.107

\* In the case of this sample the acetone was delivered straight to the flask instead of using it for the final washing of the test-tube. This represented a loss of 0.004 mg. of iron.

<i>Sample B of blood</i>	Total estimated iron in sample mg.	Known added iron mg.	Total iron minus added iron = iron in 0.2 cc. of blood taken mg.
1	0.103	0.008	0.095
2	0.098	0.003	0.095
3	0.095	0.000	0.095
4	0.103	0.006	0.097
5	0.098	0.000	0.098
6	0.099	0.004	0.095
7	0.102	0.005	0.097
8	0.096	0.000	0.096
9	0.105	0.008	0.097
10	0.099	0.002	0.097
11	0.096	0.000	0.096
12	0.103	0.007	0.096

All results are stated to the nearest significant figure.

#### SUMMARY.

A simple and rapid method for the accurate estimation of blood-iron using 0.2 cc. of blood is described. The method depends on a colorimetric comparison of the iron as ferric thiocyanate with an artificial colour standard.

My thanks are due to Prof. Raper and Prof. Craven Moore for their kind interest in this work and to Dr F. M. Irvine for one or two chemical suggestions.

#### REFERENCES.

- Berman (1918). *J. Biol. Chem.* **35**, 231.  
 Fowweather (1926). *Biochem. J.* **20**, 93.  
 Marriott and Wolf (1906). *J. Biol. Chem.* **1**, 456.  
 Walker (1925). *Analyst*, **50**, 279.  
 Wong (1923). *J. Biol. Chem.* **55**, 421.

## VII. STUDIES IN CARBOHYDRATE METABOLISM.

### II. INFLUENCE OF METHYLGLYOXAL AND OTHER POSSIBLE INTERMEDIARIES UPON INSULIN HYPOGLYCAEMIA.

By WILLIAM OGILVY KERMACK, CHARLES GEORGE LAMBIE  
(*Beit Memorial Research Fellow*), AND ROBERT HENRY SLATER.

*From the Department of Therapeutics, Edinburgh University, and the Research Laboratory of the Royal College of Physicians, Edinburgh.*

(Received November 17th, 1926.)

IN a previous communication [Kermack, Lambie and Slater, 1926], it has been shown that dihydroxyacetone when administered subcutaneously causes rabbits and mice to recover from insulin hypoglycaemia. It seemed to us that no significant difference could be discovered between the time taken for recovery after injection of dihydroxyacetone as compared with that taken after injection of glucose. In a paper by Campbell and Hepburn [1926], it has likewise been demonstrated that dihydroxyacetone causes prompt recovery from the symptoms of insulin poisoning, but these workers are of the opinion that glucose effects recovery more rapidly than does dihydroxyacetone, at least when both substances are injected intravenously. On the other hand, Hewitt and Reeves [1926], who have also confirmed the fact that dihydroxyacetone cures insulin hypoglycaemia, state that after administration of dihydroxyacetone recovery takes place in a manner identical with that seen when glucose is injected. They, like ourselves, injected the carbohydrate subcutaneously. We have now carried out further experiments to discover whether the discrepancy between our results together with those of Hewitt and Reeves on the one hand, and those of Campbell and Hepburn on the other, was due to the method of administration. We have not been able to convince ourselves that with intravenous injection there is any significant difference in the time taken for recovery. Naturally, without using very large numbers of animals it would be impossible to determine this point conclusively owing to the varying response of different animals or even of the same animal at different times.

In the paper by Hewitt and Reeves, referred to above, it is shown that glyceric aldehyde is not able to effect recovery from insulin hypoglycaemia.

We have prepared methylglyoxal by a method described by Fischer and Taube [1924, 1926] and we have tested this substance in a similar way. Briefly, we may say that the results were entirely negative and we even

received the impression that the substance possessed some toxicity. In all, seven animals were tested and in none of them did recovery take place. In each case the substance was injected subcutaneously, and usually several doses were administered. The rabbits were allowed to become completely comatose before injection of the test substance in order to minimise the risk of fallacies due to spontaneous recovery.

The following is a typical experiment.

Rabbit, female, weight 2320 g., starved 24 hours:

12.0 noon. 10 units insulin per kg.

2.50 p.m. Convulsions followed by recovery.

3.45 „ Convulsions with imperfect recovery.

4.0 „ Convulsions and coma.

4.18 „ Methylglyoxal 0.7 g. per kg. subcutaneously.

4.21 „ Violent convulsions.

4.32 „ Sprawling.

4.41 „ Convulsion, deeply comatose.

4.44 „ 0.7 g. methylglyoxal per kg.

4.48 „ Deeply comatose, lying on side apparently moribund, rapid respiration.

4.55 „ Violent convulsion.

5.5 „ Dihydroxyacetone 0.7 g. per kg. subcutaneously.

5.10 „ Sitting up in normal position.

Recovery complete and permanent.

Three of the animals to which large quantities of methylglyoxal were given, failed to recover even after subsequent injection of glucose or dihydroxyacetone. It is because of this and the peculiar deep breathing observed in these hypoglycaemic rabbits after repeated large doses of methylglyoxal, that we are led to believe that it possesses some toxic action.

A note may be added here on the preparation of methylglyoxal from dihydroxyacetone.

Experiments carried out according to the instructions of Fischer and Taube in which dihydroxyacetone (5.0 g.) and phosphorus pentoxide (15.0 g.) were thoroughly mixed and very gently heated until the reaction proceeded spontaneously, the product being condensed by liquid air or by a good freezing mixture, did not give yields as good as those claimed by these authors. The product, the yield of which varied from 1–2 g., polymerised very rapidly and became insoluble in water and so had to be mixed with an equal volume of water very soon after the distillation. If the quantity of phosphorus pentoxide was reduced, the yield became greater (up to 2.6 g.), but the product obtained was less pure, as shown by the yield of di-semicarbazone (m.p. 253°), obtained from a weighed quantity.

For instance, the products from two experiments using the larger quantity of phosphorus pentoxide gave 96 % and 99 % respectively of the theoretical yield of semicarbazone, whilst with the smaller quantity only 60.5 % of the



theoretical yield was obtained. The material obtained by the use of the smaller quantity of phosphorus pentoxide did not become vitreous as the result of polymerisation as did the other, but only thickened to a very viscous consistency.

Since either dihydroxyacetone or methylglyoxal might conceivably form lactic acid in the animal body, while the latter might either undergo complete combustion or form glucose, it was considered of interest to investigate further the influence of this acid upon insulin hypoglycaemia. We entirely failed to effect recovery or to prevent convulsions, either by subcutaneous or by intravenous injection of the substance. Our experiments therefore confirm the impression gained by Noble and Macleod [1923] who, while not coming to any final conclusion, did not obtain evidence of recovery.

Glycerol also failed to produce recovery or to prevent convulsions, even when large quantities were injected intravenously. This agrees with the results of Noble and Macleod [1923], although it should be noted that Voegtlin, Dunn and Thompson [1924, 1925], claim that glycerol administered intraperitoneally or by mouth protects mice from insulin hypoglycaemia and causes a rise in the blood-sugar of fasting rabbits. If it is active at all, it is certainly, in our experience, in no way comparable to either dihydroxyacetone or glucose.

Sodium pyruvate, like methylglyoxal, so far from removing the symptoms of hypoglycaemia, appeared to have a distinct toxic action, which, in some animals interfered with the recovery by glucose or dihydroxyacetone.

During the present work, sodium citrate and rhamnose were also tested, in each case with negative results.

#### DISCUSSION.

From the above, it is evident that there can be no comparison between dihydroxyacetone and any of the other assumed intermediaries in carbohydrate metabolism containing three carbon atoms, namely, methylglyoxal, glyceric aldehyde, glycerol, lactic acid and sodium pyruvate.

In our previous communication we left it an open question as to whether dihydroxyacetone acted by being converted into glucose or whether it could be directly oxidised, although we came to the conclusion that the balance of evidence was in favour of the latter assumption. Since this communication was written, a paper has appeared by Campbell, Fletcher, Hepburn and Markowitz [1926] in which the conclusion is reached, from a number of different lines of investigation, that dihydroxyacetone is apparently completely converted into glucose as the first step in its metabolism in the animal body. Nevertheless, we consider that such a definite conclusion is not justified. There is, we think, a considerable body of evidence to show that dihydroxyacetone may be metabolised more rapidly than glucose. Firstly, the blood-sugar curves reported by Isaac and Adler [1924], Rabinowitch [1925, 1, 2], Mason [1926, 1] and ourselves all indicate that dihydroxyacetone does not

cause an increase in the blood-sugar comparable to that produced by an equal amount of glucose. Even Campbell [1926] found very little rise in the blood-sugar curve after dihydroxyacetone although, curiously, he got very little rise in his control experiments with glucose. Secondly, Isaac and Adler [1924] have brought evidence to show that dihydroxyacetone can form glycogen with greater ease than can glucose. Thirdly, the respiratory metabolism experiments of Mason [1926, 2] indicate that dihydroxyacetone is oxidised more easily and more completely than is glucose in the normal individual and to a less extent in the diabetic<sup>1</sup>. Fourthly, in the perfusion experiment upon the eviscerated and decerebrate cat reported in our first communication, it was shown that dihydroxyacetone disappears from the blood more rapidly than glucose when perfused at the same rate, even although the liver is out of the circulation.

The problem is a difficult one, but it is not easy to reconcile these observations with the conclusions reached by Campbell, Fletcher, Hepburn and Markowitz. Particularly when we consider the greater ease with which dihydroxyacetone can be oxidised *in vitro* as compared with glucose does it seem probable that it can undergo, to some extent at least, direct oxidation. Campbell, Fletcher *et al.* found that in the completely depancreatized dog dihydroxyacetone was not only converted into glucose and quantitatively excreted as such, but that the elimination was as rapid as if an equivalent quantity of glucose had been administered. We have examined two cases of complete diabetes in the human subject, that is to say, patients who were excreting more sugar than they were taking in. In such clinical experiments it is difficult to obtain clear cut results but in general our conclusion was that the dihydroxyacetone administered was almost quantitatively excreted as glucose. Incidentally, it was confirmed by polariscopic examination that the reducing substance in the urine was actually dextrose.

It seems then that in extreme diabetes dihydroxyacetone may be converted rapidly into glucose, whereas, where insulin is available, it is more easily utilised than glucose. This conclusion is confirmed by the observations of Mason [1926, 2], who found that in diabetics dihydroxyacetone did not exhibit as great a difference in its rate of metabolism when compared with glucose as was found in the case of normal individuals.

In order to explain this result we venture to suggest the hypothesis that an equilibrium exists in the body between dihydroxyacetone and glucose (similar to that which appears to exist *in vitro* in weak alkaline solution), such that in diabetes or in the absence of insulin the tendency is in the direction dihydroxyacetone to glucose and in the opposite direction when there are inadequate supplies of insulin. Administration of excess of dihydroxy-

<sup>1</sup> [Note added January 1, 1927.] Since the present paper was submitted for publication, the results of Mason have been amply confirmed by one of us (C. G. L.) and it has further been shown, by intravenous injection, that greater and more rapid increase in metabolism after dihydroxyacetone, as compared with glucose, is not due to its more rapid absorption. These results will be published fully elsewhere.

acetone, even in the presence of insulin, would by mass action give rise to a transient hyperglycaemia.

Dihydroxyacetone will naturally be in equilibrium with glycerol and with methylglyoxal, and through the latter with lactic acid and alanine. If dihydroxyacetone is being rapidly converted into glucose, as in the diabetic organism, not only will all these substances tend to produce glucose, but also compounds such as propionic acid and fatty acids containing an odd number of carbon atoms as well as certain amino-acids which ultimately on degradation give rise to fragments containing three carbon atoms. On the other hand, after administration of dihydroxyacetone, an increase in the excretion of lactic acid is to be expected, as this is a comparatively stable product in equilibrium with dihydroxyacetone through methylglyoxal, and it has been shown, in fact, by Isaac and Adler [1924] and by Mason [1926, 1, 2] that there is an increase in the production of lactic acid under these conditions. Again, it has been shown by Smedley MacLean and Hoffert [1926] that in yeast the probability is that fat is produced directly from at least three glucose molecules and, if this applies to the animal organism, the tendency often observed in early diabetics to the deposition of fat would be simply due to the mass effect of the large amount of glucose formed and its non-conversion into dihydroxyacetone. Naturally, the fat deposits disappear when the organism becomes increasingly dependent upon these stores of fat as a source of energy. The glycerol formed from their catabolism would then go through dihydroxyacetone into glucose and, of course, it is well known that glycerol yields glucose quantitatively in the complete diabetic.

It at first seems an important objection to this view that dihydroxyacetone exists, if it exists at all, in the blood stream in a concentration of at any rate less than 1 in 20,000 (human), to 1 in 10,000 (rabbit). However, if dihydroxyacetone is extraordinarily easily oxidised and if the conversion of glucose into dihydroxyacetone under the influence of insulin only takes place comparatively slowly as compared with the rate of oxidation of dihydroxyacetone, and further when it is remembered that the site of the action of insulin is not in the blood but in the tissues [Eadie, Macleod and Noble, 1923; Burn and Dale, 1925; Lambie, 1926], such a low concentration of dihydroxyacetone in the blood stream is only to be expected.

If the above hypothesis were true, the effect of dihydroxyacetone in causing animals to recover from insulin hypoglycaemia would be that it is directly oxidised, thereby supplying energy for the activity of the nerve cells. The absence of effect in the case of any of the other supposed intermediaries would be due to the fact that they can neither be themselves oxidised in such a way that their energy can be utilised in cellular metabolism nor converted into dihydroxyacetone at a sufficient rate. Presumably the only substances which could be so converted would be glucose, fructose and the few substances which cause recovery from insulin hypoglycaemia.

That glucose and dihydroxyacetone appear to effect recovery in the same

time may be due to the fact that the limiting factor is not simply the rate at which a sufficient quantity of dihydroxyacetone is brought in contact with the tissues, whether from glucose or directly, but the time taken for the nerve and muscle cells to recover from the secondary effects of the hypoglycaemic state.

#### SUMMARY.

(1) No significant difference could be observed in the time taken to recover from insulin hypoglycaemia following intravenous dihydroxyacetone administration as compared with glucose.

(2) Methylglyoxal and sodium pyruvate fail to cause animals to recover from insulin hypoglycaemia and appear to have a toxic action.

(3) Negative results were also obtained with sodium lactate, glycerol, sodium citrate and rhamnose.

#### REFERENCES.

- Burn and Dale (1925). *J. Physiol.* **59**, 164.  
Campbell (1926). *J. Biol. Chem.* **67**, 59.  
Campbell, Fletcher, Hepburn and Markowitz (1926). *J. Biol. Chem.* **67**, lvii.  
Campbell and Hepburn (1926). *J. Biol. Chem.* **68**, 575.  
Eadie, Macleod and Noble (1923). *Amer. J. Physiol.* **65**, 462.  
Fischer and Taube (1924). *Ber. deutsch. chem. Ges.* **57**, 1502.  
——— (1926). *Ber. deutsch. chem. Ges.* **59**, 857.  
Hewitt and Reeves (1926). *Lancet*, i, 703.  
Isaac and Adler (1924). *Klin. Woch.* **3**, 1208.  
Kermack, Lambie and Slater (1926). *Biochem. J.* **20**, 486.  
Lambie (1926). *Brit. J. Exp. Path.* **7**, 22.  
Mason (1926, 1). *Canad. Med. Assoc. J.* **16**, 367.  
——— (1926, 2). *J. Clin. Invest.* **2**, 521, 533.  
Noble and Macleod (1923). *Amer. J. Physiol.* **64**, 547.  
Rabinowitch (1925, 1). *J. Biol. Chem.* **65**, 55.  
——— (1925, 2). *Canad. Med. Assoc. J.* **15**, 374.  
Smedley MacLean and Hoffert (1926). *Biochem. J.* **20**, 343.  
Voegtlin, Dunn and Thompson (1924). *Amer. J. Physiol.* **71**, 574.  
Voegtlin, Thompson and Dunn (1925). *J. Biol. Chem.* **64**, 639.

## VIII. THE HEAT-DENATURATION OF PROTEINS. PART IV.

### THE FREE BASIC AND ACIDIC GROUPS OF FRESH AND DENATURED HAEMOGLOBIN.

By PHILIP STACEY LEWIS.

*The Muspratt Laboratory of Physical and Electro-Chemistry,  
the University, Liverpool.*

(Received November 22nd, 1926.)

THE present paper<sup>1</sup> is an account of an attempt to determine directly by titration, whether any change in the total number of free basic and acidic groups takes place in the protein molecule on denaturation.

The various views of the chemical process underlying heat-denaturation may be said to lie between two extremes which are absolutely opposed. Robertson [1918] is of the opinion that this fundamental chemical reaction is one of condensation of carboxyl and amino-groups of contiguous molecules, while a further group of workers, notably Lepeschkin [1922] and Wu and Wu [1925] prefer to regard it as a mild hydrolysis.

Consequently titration, if it can be rendered sufficiently accurate, should afford a crucial test of these theories. On the view of Robertson the total number of basic and acidic groups should be diminished by denaturation; while on the opposed view this number should either increase or remain constant according to whether the linkages hydrolysed give rise to such groups, or whether, as Lepeschkin thinks, the change is more comparable with the hydrolysis of sugars.

Harris [1923, 1] in the course of an investigation on a hitherto undiscovered sulphur linking in proteins, carried out formaldehyde titrations on fresh and denatured egg-albumin and came to the conclusion that no detectable change occurred on denaturation.

It may be noted in this connection that Sørensen and Jurgensen [1910] showed that on continued heating of egg-albumin (up to 24 hours in a boiling water-bath) the formaldehyde titre of the solution, which falls at first owing to the removal of protein, increases towards the end of the reaction owing, they conclude, to hydrolysis followed by re-solution of the fragments resulting from the cleavage. This reaction is of course subsequent to denaturation. In the course of the same paper they say that they do not consider that any

<sup>1</sup> For the earlier papers of this series cf. Lewis [1926].

disintegration of the protein takes place on simple denaturation. The workers mentioned above as being of the opinion that heat-denaturation is a hydrolysis do not appear to have in mind such a violent reaction as that with which Sørensen and Jurgensen dealt.

In the present work the titratable amino-groups of the haemoglobin molecule have been determined before and after heat-denaturation. No change is indicated.

Investigation of the acidic groups of the molecule before and after denaturation by heat indicates that the reaction causes no detectable change in the number of acidic groups.

#### SECTION 1. THE FREE AMINO-GROUPS OF FRESH AND DENATURED HAEMOGLOBIN.

The method employed here was that used by Harris [1923, 2; 1924, 1, 2] for the titration of various amino-acids and complex ampholytes.

The method is to determine the  $p_H$  reached by the ampholyte solution on the addition of various amounts of standard acid. The same treatment is accorded to an equal bulk of the solvent used. The curve obtained in this latter case affords a blank correction curve from which it is possible to determine the amount of acid necessary to bring the solvent to any particular  $p_H$ . This is then subtracted from the amount of acid necessary to bring the ampholyte solution to the same  $p_H$ . The resulting figure is the amount of acid taken up by the ampholyte itself in moving to this  $p_H$  and is termed the "corrected titre" of the ampholyte. When these corrected titres are plotted against  $p_H$  they yield an S curve showing two regions in which it is asymptotic to the  $p_H$  axis. The first is in the initial stages of the titration and the second at the end of the titration where the curve becomes asymptotic to some particular titre. This latter value is the actual titre of the ampholyte, and is the amount of acid necessary to neutralise its basic groups.

Both with the solution and the solvent the titration is commenced from neutrality. The curve of corrected titres against  $p_H$  is termed the "titration curve" of the ampholyte.

#### *Experimental.*

In the following work each point was determined separately. That is to say that the whole titration was not carried through with one solution but a separate solution was made up for each amount of acid added. This was deemed preferable as in this manner the haemoglobin was only under the influence of the acid for 5 to 10 minutes during each determination. This tended to eliminate error due to possible denaturation by the acid.

The solutions were made up by taking 20 cc. of 1 % fresh haemoglobin each time. This was either denatured or retained as such. The denaturing was carried out by immersing a corked flask, containing the haemoglobin, in the steam from a briskly boiling water-bath for 10 minutes. The flask and

contents were subsequently cooled and every drop of condensed moisture in the flask collected back into the original liquid. A little loss of water would be bound to ensue but it would be relatively negligible. To the solutions so obtained the desired amount of acid was then added.

The  $p_H$  of each solution was then determined at  $25^\circ$  by means of the quinhydrone electrode, by measurement of the E.M.F. of a cell of the type:

Gold electrode	Solution under test saturated with quinhydrone	Saturated KCl	Normal calomel electrode
-------------------	--	------------------	-----------------------------

The solution contained 1 g. of haemoglobin and 0.054 g. of ammonium sulphate per 100 cc. The titration was performed with 0.096 N  $H_2SO_4$ . The titrations are accurate to  $\pm 0.01$  cc.

The following results were obtained. For the sake of brevity only mean  $p_H$  values are given. Each value is the mean of six independent readings, the accuracy being  $\pm 0.003 p_H$ .

Table I. *Titration of 20 cc. of 0.054 %  $(NH_4)_2SO_4$  with 0.096 N  $H_2SO_4$ .*

cc. $H_2SO_4$	0	0.02	0.03	0.05	0.20	0.30	0.50	1.00	1.50	2.00	2.50	3.00
$p_H$	6.771	4.117	3.802	3.564	3.107	2.936	2.724	2.462	2.301	2.195	2.112	2.039

From Table I a correction curve was drawn from which the values in the horizontal rows headed "blank" of the tables below are obtained.

Table II. *The titration of 20 cc. of 1 % fresh (undenatured) haemoglobin containing 0.054 % of  $(NH_4)_2SO_4$ , with 0.096 N  $H_2SO_4$  at  $25^\circ$ .*

cc. $H_2SO_4$	0	0.30	1.00	2.00	3.00	4.00	5.00	6.00
$p_H$	6.781	5.521	4.451	3.440	2.809	2.443	2.198	2.047
Blank in cc.	0	0.015	0.02	0.075	0.32	1.08	2.00	2.98
Corrected titre in cc.	0	0.285	0.98	1.925	2.68	2.92	3.00	3.02

Table III. *The titration of 20 cc. 1 % denatured haemoglobin containing 0.054 % of  $(NH_4)_2SO_4$ , with 0.096 N  $H_2SO_4$  at  $25^\circ$ .*

cc. $H_2SO_4$	0	0.30	1.00	2.00	3.00	4.00	5.00	6.00
$p_H$	6.778	5.529	4.435	3.441	2.812	2.433	2.195	2.047
Blank in cc.	0	0.015	0.02	0.075	0.31	1.09	2.00	2.98
Corrected titre in cc.	0	0.285	0.98	1.925	2.69	2.91	3.00	3.02

The titration curves derived from these tables are shown in Fig. 1. These curves show the variation of corrected titre with  $p_H$ .

It will be seen that the titration curves of fresh and denatured haemoglobin are sensibly identical. Either corresponds to a titration of 3.05 cc. of 0.096 N  $H_2SO_4$  for 20 cc. of 1 % haemoglobin. From this it follows that 100 g. of haemoglobin contain 2.05 g. of titratable nitrogen.

It is generally assumed that the  $\alpha$ -amino-groups of amino-acids are entirely utilised in linkages in the protein molecule. Consequently the titratable basic groups will be the second basic groups of the dibasic acids in the molecule. According to Van Slyke and Birchard [1913] the diamino-acids present in haemoglobin are lysine, histidine and arginine which account for 11.90, 12.69

and 7.72 % respectively of the total nitrogen of the molecule. Under the present circumstances one half of the total lysine nitrogen, one-third of the histidine nitrogen and one-quarter of the arginine nitrogen<sup>1</sup> will be titratable; *i.e.*  $(5.45 + 4.23 + 1.93) = 11.61$  % of the total nitrogen of the molecule. The total nitrogen of the haemoglobin molecule of the ox being given by Mathews [1924] as 17.70 % of the molecule, it would be expected on this basis that there would be 2.055 g. of titratable nitrogen per 100 g. of haemoglobin. This is in very good agreement with the value obtained above. It is stated with some degree of certainty that the present results point to the absence of change in the number of titratable basic groups of the haemoglobin molecule on denaturation.

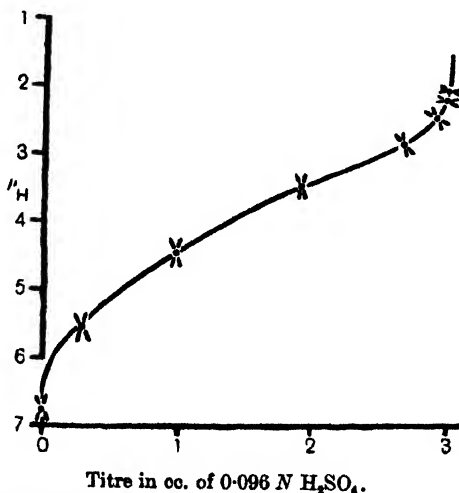


Fig. 1. Corrected titration curves of fresh and denatured haemoglobin.

• Titration of fresh haemoglobin.

X Titration of denatured haemoglobin.

Assuming the value of 16,660 as the equivalent weight of haemoglobin [Mathews, 1924] it is found that these figures correspond to 24.4 titratable nitrogen atoms per equivalent.

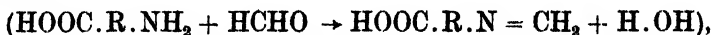
## SECTION 2. THE FREE CARBOXYL AND OTHER ACIDIC GROUPS OF FRESH AND DENATURED HAEMOGLOBIN.

In order to investigate this problem, use was made of a modification of the formaldehyde titration of Sørensen. Normally a titration of carboxyl groups has to be carried to a very alkaline end-point to obtain complete titration. In this case the correction for the dilution of the system of titrant

<sup>1</sup> Lysine, histidine and arginine contain respectively 2, 3 and 4 nitrogen atoms, of which two are titratable in each case. In lysine and arginine these two atoms are members of amino-groups and in histidine one is a member of an iminazole ring. In each case one of these two basic groups is an  $\alpha$ -amino-group and therefore assumed bound. This leaves one nitrogen atom titratable in each of these amino-acids. Hence the above fractions.



becomes undesirably large. If, however, formaldehyde be added to the protein before titration the acidic nature of the protein is much enhanced and the titration is complete at about  $p_H$  9. This is explained [see Harris, 1924, 2] as being due to the formation of methylene-imino-derivatives of the amino-acids present



which have an acidic dissociation constant approximately one thousand times that of the original protein. The addition of formaldehyde therefore forms a means of enhancing the accuracy of the titration.

Harris [1924, 2] also advocates the use of alcohol to enhance the accuracy of the titration, but this is not permissible in the present instance owing to the action of alcohol on the protein itself, which reaction is identified with heat-denaturation by Robertson.

If it is desired to perform a titration of the protein and to form an accurate estimate of the total number of acidic groups of the molecule, it is necessary to determine the  $p_H$  of successive solutions containing protein, formaldehyde, and different known amounts of alkali. Three methods of making these determinations suggest themselves, namely, the hydrogen gas electrode, the Donnan-Allmand electrode and the glass electrode. In this case the quin-hydrone electrode may not be used on account of the alkalinity of the solutions near the end-point.

Of these electrodes the Donnan-Allmand electrode was first utilised. It was not desirable that the oxyhaemoglobin should be exposed to the reducing action of the hydrogen.

The results obtained using the Donnan-Allmand electrode indicated that denatured protein binds more alkali than does fresh. In other words, that by the process of heat denaturation fresh acidic groups were uncovered. The difference, however, was but slight and the results appeared unsatisfactory. The reproducibility was poor. Furthermore the electrodes required periods up to 48 hours to come to equilibrium. In work of the present nature it is most undesirable that the protein should be exposed to the alkali for this length of time. It is almost inevitable that some hydrolytic decomposition, subsequent to denaturation proper, should have taken place during this period.

Consequently a similar series of determinations was made with the glass electrode. The method of  $p_H$  measurement adopted was that described by Mrs Kerridge [1925]. As in dealing with the glass electrode it is required to measure the E.M.F. of a cell of very high resistance, the ordinary galvanometric method is not feasible. Instead a system is substituted in which the detector is a quadrant electrometer. The electrometer used was a Lindemann electrometer as described by Lindemann and Keeley [1924]. The greatest accuracy obtained is 0.4 to 0.5 millivolt. While this may be capable of improvement the objection still remains that any electrostatic method is extraordinarily sensitive to stray fields of force and to possible leakages of charge. It was found necessary to work within a hollow earthed conductor, and to take the

greatest care that all apparatus was well insulated. As insulating medium amberite was utilised as suggested by Mrs Kerridge. Even so leakage of charge was apt to become a troublesome factor in wet weather. On the whole, however, this seems to be the most satisfactory method of measuring the  $p_H$  of alkaline solutions. Its two great advantages are rapidity and a sensible absence of diffusion.

The solutions were made up by taking 20 cc. of 2 % fresh haemoglobin each time. This was either denatured or retained as such. To this solution 8 cc. of 10 % formaldehyde, previously adjusted to  $p_H$  7.0, were added. Following this, the known amount of NaOH was added and the  $p_H$  determined. The results are given below. The "blank" determinations are not recorded as they have no bearing on the point at issue.

Here again, for the sake of brevity, detailed readings are omitted. The readings in Table IV are the mean of five readings in each case with independent solutions. The titres are accurate to 0.01 cc. The  $p_H$  measurements are accurate to  $\pm 0.006 p_H$ .

Table IV. *Formaldehyde titration of 20 cc. 2.0 % haemoglobin containing 0.3 %  $(NH_4)_2SO_4$  with 8 cc. formaldehyde added. Temperature 18°. Titrant 0.360 N NaOH.*

Titre	0	0.20	0.40	0.60	1.00	1.20	1.60	2.00	2.60	3.00
$p_H$ of haemo- (Fresh	7.18	7.06	7.98	8.25	8.66	8.81	9.15	9.50	10.05	10.38
globin (Denatured	7.18	7.66	7.97	8.25	8.66	8.80	9.15	9.50	10.05	10.38

From the above figures it will be seen that there is no discernible difference between the fresh and denatured material.

These results are not so accurate as those of Section 1. On the present results the writer is only prepared to state that if there is a change in the number of free acidic groups in the molecule it is too small to be detected, at least by the present means. Taken in conjunction with the results of Section 1 it becomes quite probable that actually there is no change on denaturation.

The present results are definitely opposed to the view adopted by Robertson [1918], namely that denaturation entails the chemical condensation of free amino-groups in adjacent molecules.

Lepeschkin [1922] has suggested that the reaction involved is a hydrolysis of the same nature as that by which a polysaccharide is broken up into its constituent units. The results embodied in the present paper point to a grouping of some such character being involved. Certainly the linkage involved cannot be the peptide link ( $-\text{CONH}-$ ) which, on *a priori* grounds might have been thought important for such a reaction.

It is of interest here to record a titration in full and thus to determine the number of free acidic groups in one equivalent weight of haemoglobin. The necessary blank measurements have been made and the corrected titration curve drawn as shown in Fig. 2.

The measurements are recorded below.

Table V. *Titration of 20 cc. 0.3 % ammonium sulphate solution to which had been added 8 cc. of 10 % formaldehyde. Titrant 0.360 N NaOH.*

Titre	0	0.50	1.00	2.00	2.50
p <sub>H</sub>	7.20	8.51	9.47	10.42	10.64

From Table V a correction curve was drawn and from it the values in the horizontal row headed "blank" in the table below were obtained.

Table VI. *Corrected titrations of 2 % fresh haemoglobin with 0.360 N NaOH.*

cc. NaOH	0%	0.20	0.40	0.60	1.00	1.20	1.60	2.00	2.80	3.00
p <sub>H</sub>	7.18	7.66	7.98	8.25	8.68	8.81	9.15	9.50	10.05	10.38
Blank	0	0.17	0.30	0.40	0.55	0.60	0.78	1.03	1.55	1.94
Corrected titre	0	0.03	0.10	0.20	0.45	0.60	0.82	0.97	1.05	1.06

From the corrected titration curve (Fig. 2) it will be seen that the 0.4 g. of haemoglobin present in 20 cc. of 2 % solution is neutralised by 1.06 cc. of 0.360 N NaOH. From this it is calculated that in the equivalent of haemoglobin (mass 16,660) there are 16 free acidic groups.

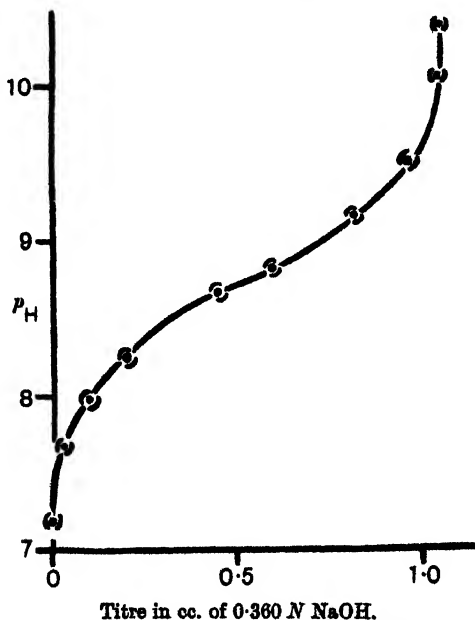


Fig. 2. Formaldehyde titration of 2 % fresh haemoglobin.

#### SUMMARY.

(1) It is found that in the equivalent of haemoglobin (taken to have mass 16,660) there are 24 or 25 free basic groups and 16 free acidic groups.

(2) Furthermore it is found that heat-denaturation is without effect on the number of free basic groups and without detectable effect on the number of free acidic groups of the haemoglobin molecule.

(3) From these results the conclusion is drawn that denaturation involves the hydrolysis of linkages of a character analogous to those involved in the hydrolysis of polysaccharides, as distinct from rupture of the polypeptide link ( $-\text{CONH}-$ ). This conclusion is in agreement with earlier papers by the writer.

In conclusion, the writer desires to express his indebtedness to the Department of Scientific and Industrial Research for a grant which enabled the present research to be carried out. Further, the writer would like to acknowledge the benefit of communications with Dr L. J. Harris, and of the helpful advice of Prof. W. C. M. Lewis, under whom these investigations have been made.

## REFERENCES.

- Harris (1923, 1). *Proc. Roy. Soc. Lond. B.* **94**, 426.  
—— (1923, 2). *J. Chem. Soc.* **123**, 3294.  
—— (1924, 1, 2). *Proc. Roy. Soc. Lond. B.* **95**, 440, 500.  
Kerridge (1925). *Biochem. J.* **19**, 611.  
Lepeschkin (1922). *Kolloid Z.* **31**, 3342.  
Lewis (1926). *Biochem. J.* **20**, 965, 978, 984.  
Lindemann and Keeley (1924). *Phil. Mag.* **47**, 577.  
Mathews (1924). *Physiological Chemistry*, 3rd ed., p. 110.  
Robertson (1918). *The physical Chemistry of Proteins*.  
Van Slyke and Birchard (1913). *J. Biol. Chem.* **16**, 539.  
Sørensen and Jurgensen (1910). *Compt. Rend. Lab. Carlsberg*, **10**, 49.  
Wu and Wu (1925). *J. Biol. Chem.* **44**, 369.

## IX. THE FATE OF DESOXY-GLUCOSE IN THE RABBIT.

By LEWIS BLAND WINTER.

*From the Biochemical Laboratory, Cambridge.*

*(Received November 23rd, 1926.)*

It has been shown [Winter, 1926] that the injection of glugal into rabbits during insulin convulsions brings about complete recovery to normal with raising of the blood-sugar. The possible lines on which glugal is effective in restoring the animal may be, (1) direct oxidation, (2) conversion into glucose or mannose, (3) formation of desoxy-glucose, which is a reducing sugar, but which differs from glucose in that two H atoms are attached to the second carbon. The possibility of the metabolism of desoxy-glucose has been tested by injection into rabbits in convulsions. The substance was prepared by the method of Bergmann, Schotte, and Lechinsky [1922], by the action of acid on glugal. The glugal was however allowed to crystallise, and was drained on a porous tile before treatment with acid. A few grams of the diphenylhydrazone [Bergmann and Schotte, 1921] were first prepared, and the compound decomposed with benzaldehyde. By this means a crystalline sample of the sugar was readily obtained, and this was used for nucleating the main preparation. The final product gave  $[\alpha]_{5461} = + 59.5^\circ$ . The sugar was dissolved in a little water, and injected into the animals when the convulsions were well established. In one case a temporary improvement in the condition of the animal was noted, soon followed by a relapse. In no case has complete recovery been effected, though the subsequent administration of glucose will restore the animal permanently, showing that the desoxy-glucose has no toxic effect. There is a rise in the value for the blood-sugar following the injection of desoxy-glucose, but since there is little alteration in the condition of the animal it is probable that the increased amount of reducing substance is almost entirely due to the presence of the unaltered compound, and that the substance is not capable of metabolism by the tissues. The urine is found to give a green pine-shaving reaction, and in the HCl modification of the  $\alpha$ -naphthol test a purple colour develops as soon as the mixture is warmed. It has been found that glugal and desoxy-glucose behave like the pentoses in respect to this reaction. There is however no possibility of confusion if pentoses are estimated in the presence of glugal, since the distillation of 0.03-0.05 g. of glugal with hydrochloric acid gave no weighable precipitate on adding phloroglucinol and allowing to stand. The colour reaction with

$\alpha$ -naphthol is evidently not due to the formation of furfural. The urine after shaking with charcoal reduced Fehling's solution vigorously. In one case a Wood-Ost estimation, which is less likely to be affected by nitrogenous compounds, indicated 0.33 % of sugar reckoned as glucose, following the administration of desoxy-glucose. Since the animal had not recovered from the insulin convulsions, it is likely that the main reduction was due to the injected sugar. The excretion of glucose during insulin hypoglycaemia is inconceivable. No precipitate of diphenylhydrazone could however be obtained when an alcoholic solution of diphenylhydrazone was added to the urine, the mixture being filtered and allowed to stand after shaking for 4 hours.

Weight kg.	Time	Protocols	Blood-sugar %
1.5	9.50	—	0.085
	10.00	3 mg. ins. HCl	—
	2.25	2 mg. ins. HCl	—
	3.20	—	0.059
	6.00	Convulsions	0.050
	6.20	0.5 g. desoxy-glucose	—
	7.45	Still collapsed. 0.5 g. desoxy-glucose	—
	9.15	Violent convulsions	0.060
	9.30	2 g. glucose	—
	10.30	Appeared normal	0.083
	10.00	—	0.091
	10.10	3 mg. ins. HCl	—
1.1	1.00	Convulsions	0.034
	1.20	1.0 g. desoxy-glucose	—
	2.00	Better, able to eat	—
	2.30	Collapsed	—
	2.45	1.0 g. desoxy-glucose	—
	4.45	—	0.11
	6.45	Still collapsed	0.062
	8.30	Animal died	—
	9.45	—	0.089
	9.55	3 mg. ins. HCl	—
1.1	12.30	Convulsions	0.041
	12.45	1.0 g. desoxy-glucose	—
	2.45	Collapsed	0.058
	4.00	Animal died	—

## REFERENCES.

- Bergmann and Schotte (1921). *Ber. deutsch. chem. Ges.* **54**, 440.  
 Bergmann, Schotte and Lechinsky (1922). *Ber. deutsch. chem. Ges.* **56**, 158.  
 Winter (1926). *Biochem. J.* **20**, 668.

# X. STUDIES ON THE KINETICS OF HAEMOLYTIC SYSTEMS.

## II. THE SERIES OF RYVOSH.

By ERIC PONDER.

*From the Department of Physiology, Edinburgh University.*

*(Received November 23rd, 1926.)*

THE series of Ryvosh is concerned with the order in which the erythrocytes of the mammalia may be placed with respect to haemolysis by saponin and hypotonic saline respectively. Ryvosh [1907] places the types of cell in the following order, the most resistant species occurring first.

*Saponin.* Sheep, goat, ox, cat, grey mouse, pig, grey rat, dog, white rat, rabbit, guinea-pig.

*Hypotonic saline.* Guinea-pig, white rat, dog, grey rat, rabbit, pig, grey mouse, cat, ox, goat, sheep.

It will be observed that the order of resistance to saponin is the reverse of that to hypotonic saline, with the rabbit forming an exception.

This antagonism between the resistance to saponin and that to hypotonic saline has often been remarked upon, and various reasons have been put forward as to why it should exist. In particular, Orahovats [1926] has recently referred to the series in connection with his researches on the resistance of red cells in splenic blood, and as a result of these researches the subject assumes considerable importance. The results of Ryvosh having been obtained by methods which are open to considerable objection, we propose to investigate the matter afresh by a more satisfactory experimental procedure.

The objections to the original methods are principally the three following.

(1) Ryvosh added defibrinated blood to his solutions of NaCl and of saponin. The haemolytic systems accordingly contained serum, the presence of which renders the measurements of resistance to saponin unreliable, for serum itself inhibits saponin haemolysis [Ponder, 1923].

(2) The amount of blood thus added was in all cases such as to produce a 1 % suspension of cells. Now for experiments such as these, we can prepare suspensions in one of three ways. (a) We may have, in the case of the suspension from each animal, 1 cc. containing the cells from a constant volume of blood. This is the manner in which the suspensions used by Ryvosh were prepared. (b) We may arrange the suspensions so that each contains, in 1 cc., a constant number of cells. (c) We can make the suspensions so that 1 cc. of each contains a number of cells which present constant surface. To

decide which of these three alternative methods is the best is a difficult matter. The first is the most arbitrary, and in the case of saponin haemolysis the third probably the best, since the kinetics of saponin haemolysis are described by expressions which contain terms relating to the surface of the cells [Ponder, 1926, 1]. In this paper we shall, however, consider the resistances of suspensions prepared according to methods (a), (b) and (c), in preference to selecting one of these methods to the exclusion of the others.

In the case of haemolysis by hypotonic saline this difficulty does not arise, since the mechanism of the haemolysis is entirely different from that of saponin lysis, and since in the case of haemolysis by hypotonic saline we are not dealing with a process in which lysin is used up. We can accordingly compare, as Ryvosh does, suspensions containing the cells from a constant volume of blood.

(3) In the series of Ryvosh we have the members arranged merely according to order, and the order of resistance to saponin the reverse of that to hypotonic saline. When we compare the series for saponin with that for hypotonic saline and arrive at the conclusion that the one is the reverse of the other, we imply that if the members of the series are correlated according to rank, we shall find a coefficient of rank correlation of  $-1$ . It is well known, however, that the significance of such a value of a coefficient of rank correlation may be very much less than it appears to be, for the coefficient of rank correlation may differ very considerably from the coefficient of real correlation. We therefore seek to replace the arrangement by order by an arrangement according to some absolute measurement, so that we may be able to find the coefficient of real correlation, the value of which is the only reliable guide.

#### METHODS.

*Saponin haemolysis.* The method used for the measurement of the relative resistance of various types of cell to saponin haemolysis has been fully described in previous papers [Ponder, 1926, 1, 2, 3]. In brief, it consists of plotting the time-dilution curves for the action of saponin on some type of cell selected as a standard—in these experiments, the cells of man—and on the type of cell whose relative resistance is required. The asymptotes of the two curves are found, and the concentrations of lysin corresponding to these asymptotes written down; the division of the one figure by the other supplies a resistance constant  $R$ , the magnitude of which gives the resistance of the one type of cell in terms of that of the standard type. The method is somewhat involved, but exceedingly accurate, and has the advantage that it expresses the resistance as the ratio of two absolute quantities. Moreover,  $R$  is constant not only for the concentrations corresponding to the asymptotes, but for all other concentrations of lysin, and thus the disadvantage of employing an arbitrarily selected concentration of lysin, or of estimating haemolysis after an arbitrarily selected time, is overcome.



All measurements are carried out at 25°; the saponin used is Merck's pure white saponin. The cell suspensions are prepared in the manner described in previous papers, and suitably diluted so as to contain either (a) the cells from a constant volume of the animal's blood, (b) a constant number of cells, or (c) a number of cells which present a constant surface. The dilution necessary is to be found from the figure for the red cell count per mm.<sup>3</sup> of blood, and the figure for the surface of the cell as calculated from photographic measurements.

*Hypotonic saline.* In order to ascertain the resistance of different types of cell to hypotonic saline, a special series of solutions of varying tonicity is prepared. These are adjusted so as to give convenient tonicities when 0.2 cc. of suspension—consisting of cells suspended in 0.8 % NaCl—is added to 1.8 cc. of each solution. The following series is suitable.

cc. of 0.8 % NaCl	Resulting tonicity g. NaCl %	cc. of 0.8 % NaCl	Resulting tonicity g. NaCl %
0	0.08	44.4	0.40
5.5	0.12	50.0	0.44
11.1	0.16	55.5	0.48
17.5	0.20	61.1	0.52
22.2	0.24	66.6	0.56
27.5	0.28	72.0	0.60
33.3	0.32	77.7	0.64
39.0	0.36	83.3	0.68

To the quantity of 0.8 % NaCl, as shown in this table, there is added distilled water to 100 cc.; the result is a solution which gives, when to 1.8 cc. of it is added 0.2 cc. of the suspension in 0.8 % NaCl, a tonicity as shown by the corresponding figure in the table. The use of these solutions is much more convenient than is the drop method of Hamburger. If tonicities intermediate to those given in the table are required, they are easily obtained by mixing two of the solutions in the proper quantities.

The method used is to add to 1.8 cc. of each of the solutions 0.2 cc. of a suspension prepared by suspending the thrice washed cells from 1 cc. of blood in 20 cc. of 0.8 % NaCl, the entire experiment being carried out at 25°. After 60 minutes the tubes are examined, and the greatest tonicity which brings about complete haemolysis is noted. Solutions which give tonicities near this figure are now prepared, and with them the experiment is repeated, so as to determine the greatest tonicity which gives complete haemolysis to 0.01 % of NaCl.

Certain observations on this technique have to be made. (1) Brinkmann [1922] has pointed out that, for tonicity experiments in general, it is better to use solutions containing NaCl, KCl, CaCl<sub>2</sub>, and NaHCO<sub>3</sub>, than to use solutions of pure NaCl. The tonicity is varied by altering the NaCl content, the other components being kept the same. In this series of experiments we do not follow Brinkmann's suggestion for the following reason. It is well known that the most suitable balance of NaCl, KCl and CaCl<sub>2</sub> varies greatly for the erythrocytes of the different mammals; a solution such as Brinkmann recommends, although excellent for the study of the resistance of one particular

type of cell, will accordingly be unsuitable when cells of different species are to be compared, for the particular NaCl, KCl and  $\text{CaCl}_2$  balance which is suitable for one animal may be very unsuitable for another, and thus we should obtain effects not directly connected with that of hypotonicity. We accordingly use pure NaCl. (2) We measure the resistance by the greatest tonicity which will complete haemolysis in 60 minutes in order to proceed in the same general way to that by which we measure the resistance to saponin. In the latter case we determine the asymptote of a time-dilution curve; here too, we determine what corresponds to an asymptote, for we imagine, although we do not actually do it, that we are plotting tonicity against the time taken to produce complete lysis, and we select that tonicity which produces complete lysis in 60 minutes—a time which, in these experiments, is as near infinity as we require. The justification of this step is that a tonicity a little greater than that selected would never complete haemolysis at all. (3) It is very important to remember that in these experiments, as in those determining the resistance to saponin, we are determining the resistance of the most resistant cells of the sample, and not of the cells of average resistance. The determination of the resistance of the average cell of the sample would be an exceedingly difficult matter. The importance of this point is that when we find a series in which the various cells may be placed with respect to their resistance, this series only holds for the most resistant cells; unless the standard deviation is the same for every suspension, the series may not hold for the average cells of the various suspensions.

*Coefficients of correlation.* These are calculated in the usual way. The coefficient of real correlation,  $r$ , is given by

$$\frac{S(xy) - Nd_1d_2}{\sqrt{\{S(x^2) - Nd_1^2\}} \sqrt{\{S(y^2) - Nd_2^2\}}},$$

where  $x$  and  $y$  are measured from points distant  $d_1$  and  $d_2$  from their respective means, and the coefficient of rank correlation,  $\rho$ , by

$$1 - \frac{S(g^2)}{\frac{1}{3}N(N^2-1)},$$

where  $S(g)$  denotes the sum of the gains in rank of the second series over the first.

## RESULTS.

### 1. *Suspensions containing cells from constant volume of blood.*

Each suspension was prepared so that the thrice washed cells from 1 cc. of the blood of the animal were finally suspended in 20 cc. of saline. The resistances to saponin and to hypotonic saline were determined by the above methods; in the table which shows the results the resistance to saponin is indicated by the figure for the asymptote of the time-dilution curve, the dilution of lysin being converted, for convenience in calculation, into the corresponding concentration of lysin in mg. The value of  $R$  is also given, the

cells of man being taken as the arbitrary standard. The resistance to hypotonic saline is given as the percentage of NaCl which gives the greatest tonicity capable of producing complete lysis in 60 minutes.

Table I.

Animal	Resistances			Rank	
	Saponin		Saline		
	Asymptote	R		Saponin	Saline
Man	0.030	1.0	0.35	3	1
Guinea-pig	0.033	1.1	0.40	4	2
Rat	0.021	0.7	0.40	2	3
Rabbit	0.012	0.4	0.42	1	4
Dog	0.036	1.2	0.44	5	5
Pig	0.039	1.3	0.45	6	6
Cat	0.063	2.1	0.48	7	7
Ox	0.198	6.6	0.49	9	8
Goat	0.075	2.5	0.52	8	9
Sheep	0.210	7.0	0.56	10	10

An inspection of this table will show that the different types of cell fall, as regards their resistance to hypotonic saline, in Ryvosh's series. As regards their resistance to saponin, they fall in a series which is nearly the reverse of the first, exceptions being provided by the goat, rabbit, and rat. Ryvosh gives the rabbit as the only animal which falls out of place in the series, for he finds the resistance of the cells of the goat greater than that of ox cells to saponin; in general, however, the order in which Ryvosh places the different types of cell is confirmed.

We now look at the correlation coefficients. Taking the series as given by Ryvosh, and correlating according to rank, we obtain a value of  $\rho$  of  $-0.94$ . This value is very high, as might be expected from the fact that the order for resistance to saponin is the reverse of that for resistance to hypotonic saline, with the one exception of the rabbit. Taking next the series as found in Table I, and correlating according to rank, we get a value of  $\rho$  of  $0.88$ , to which a negative sign must be prefixed, as the figure giving the resistance to saline becomes greater as the resistance becomes less. Next taking the correlation of the resistances as measured in absolute quantities, we obtain a much lower figure for  $r$ , the coefficient of real correlation, for it works out as  $0.75$ , to which, again, a negative sign is to be attached.

## 2. Suspensions containing a constant number of cells.

In this series of experiments, each suspension was so prepared as to contain  $2.5 \times 10^8$  cells in 1 cc. In the table showing the results, the same arrangement as in Table I is adopted, and there is added a column to show the number of cells present in 1 mm.<sup>3</sup> of the blood of each animal examined. This last figure was obtained by a red cell count made in the usual way.

Table II.

Animal	Cells per mm. <sup>3</sup> × 10 <sup>-6</sup>	Resistances			Rank	
		Asymptote	R	Saline	Saponin	Saline
Man	5.0	0.030	1.0	0.35	3	1
Guinea-pig	5.0	0.033	1.1	0.40	5	2
Rat	7.0	0.019	0.6	0.40	2	3
Rabbit	6.0	0.011	0.4	0.42	1	4
Dog	7.0	0.032	1.1	0.44	4	5
Pig	5.0	0.039	1.3	0.45	6	6
Cat	6.0	0.057	1.9	0.48	8	7
Ox	6.0	0.180	6.0	0.49	10	8
Goat	15.0	0.045	1.5	0.52	7	9
Sheep	10.0	0.140	4.6	0.56	9	10

The change in the method of preparing the suspension, it will be observed, causes a very considerable difference in the order of resistance to saponin. The coefficient of rank correlation now works out at  $-0.80$ , and the coefficient of real correlation at no greater figure than  $-0.68$ .

### 3. *Suspensions presenting constant area.*

In order to prepare these suspensions, one has to take account of the number of cells per mm.<sup>3</sup> of blood, and the surface area of each cell. The first figure can be found by a count in the usual way; the second must be determined by calculation from the figures for the diameter and thickness of the cells. These can be obtained from photographic measurements of the erythrocytes suspended in plasma; the surface area is then calculated from the expression

$$\text{Area} = 2\pi A^2 + 2\pi AB \frac{\sinh^{-1} e}{e}.$$

$A$  and  $B$  are the semi-axes major and minor of the cell, the biconcavities being imagined to be turned inside out, so that the cell assumes the form of a spheroid. The eccentricity about the minor axis is  $e$ , and it may be pointed out that the part of the expression,  $\sinh^{-1} e/e$ , is remarkably constant for all the cells of the mammalia, being equal to  $0.6$ .

Multiplying the number of cells per mm.<sup>3</sup> of blood by the figure for the area of each cell in  $\mu^2$  gives a figure which is proportional to the surface area presented by a suspension containing the cells from 1 cc. of the animal's blood. This figure may be compared with a similar figure for a suspension of human cells, which it is convenient to take as an arbitrary standard; in this way a series of suspensions from the blood of different animals can be prepared, each presenting the same surface to the lysis. In this series of experiments, the surface presented by the cells of 1 cc. of any of the suspensions used was  $30 \times 10^9 \mu^2$ .

In Table III the value of a constant  $S$ , denoting the ratio of the surface presented by the cells in 1 cc. of the animal's blood to the surface presented by the cells in 1 cc. of human blood, is given in order that the strength of the various suspensions may be readily compared.

Table III.

Animal	<i>S</i>	Resistances				Rank	
		Saponin		Saline			
		Asymptote	<i>R</i>			Saponin	Saline
Man	1.0	0.030	1.0	0.35		3	1
Guinea-pig	0.8	0.039	1.3	0.40		5	2
Rat	1.0	0.021	0.7	0.40		2	3
Rabbit	0.95	0.012	0.4	0.42		1	4
Dog	1.0	0.036	1.2	0.44		4	5
Pig	0.53	0.050	1.7	0.45		6	6
Cat	0.62	0.085	2.8	0.48		7	7
Ox	0.66	0.230	7.7	0.49		9	8
Goat	0.72	0.090	3.0	0.52		8	9
Sheep	0.7	0.250	8.3	0.56		10	10

The order of resistance to saponin under the conditions of this experiment is very nearly the same as that shown in Table I, for there is a tendency for the greater number of cells, as found in the sheep and goat, to cancel out with a small figure for the surface area.

The coefficients of correlation work out as follows:  $\rho = -0.85$ ,  $r = -0.76$ . These are very close to the values obtained from Table I, and considerably higher than those obtained from Table II.

#### DISCUSSION.

Regarding the coefficients of rank correlation, we may set these aside at once. The high value obtained from Ryvosh's original series, and the not much lower value obtained from Table I, are quite misleading, and no deductions can be drawn from them except that the order in which the various types of cell fall with respect to resistance to saponin and to hypotonic saline is extremely unlikely to be brought about by mere chance. The odds against this order occurring by chance alone, are, in fact, about 6000 to 1.

We are also inclined to set aside the results of Table II, with a value of  $r$  of  $-0.68$ . There appears no good reason for comparing suspensions containing a constant number of cells, when we have a comparison between suspensions presenting constant surface to the lysin. In any case, the value of  $r$  obtained by this method of comparison is the lowest of all; if the comparison of suspensions containing a constant number of cells should be insisted upon, the remarks about to follow would apply *a fortiori*.

Tables I and II each yield a value of  $r$  of about  $-0.75$ . This is a high value for a coefficient of correlation, but not so high as to justify us in saying that there is only one factor determining the resistance—a factor which gives a high resistance to saponin and a low one to hypotonic saline. It appears permissible, however, to say that one important factor must exist, coupled with perhaps one, or more, subsidiary factors, the operation of which may affect, in one direction or the other, the operation of the primary factor.

The nature of this primary factor is a matter for interesting speculation. Port [1922] has suggested that the phosphoric acid content of the cell is the

essential factor in determining the resistance, for he observes that the order of resistance to saponin for the cells of different animals is the same as the order of phosphoric acid content as given by Abderhalden [1895], while the order of resistance to hypotonic saline is the reverse of the order of phosphoric acid content. In applying this suggestion to his own researches Orahovats apparently takes the term "phosphoric acid" to include inorganic phosphates; with this interpretation, he finds little in support of Port's suggestion.

Examining Abderhalden's table, we find four sets of figures which bear on this point; figures are given for inorganic phosphoric acid, total phosphoric acid, phosphoric acid as nuclein, and lecithin. Although only seven animals appear in common to both Abderhalden's table and Table I of this paper, a close examination of these figures throws considerable light on the suggestion of Port.

First we correlate, according to rank, the total phosphoric acid content and the resistance to saponin. This gives a coefficient of  $-0.84$ , which is very high. This total phosphoric acid, it is now to be observed, may be broken up into the inorganic and the organic phosphoric acid, for both of which figures may be obtained from Abderhalden's table. Further, the principal sources of the organic phosphoric acid are nuclein and lecithin, for both of which Abderhalden gives figures. Correlating according to rank with the resistance to saponin, we find the following coefficients:

Total phosphoric acid and resistance	...	...	$-0.83$
Inorganic phosphoric acid and resistance	...	...	$-0.52$
Organic phosphoric acid and resistance	...	...	$-0.53$
Lecithin and resistance	...	...	$-0.21$
Phosphoric acid as nuclein and resistance	...	...	$-0.89$

These being merely rank correlations, it is difficult to attach a significance to the figures, but one thing stands out clearly—the correlations are all negative, the greater content of any one of these substances being associated with the lower resistance to saponin.

This fact is very important, for it has been established with a considerable degree of certainty that saponin enters into a combination with some component of the cell, from which we should expect to find the amount of that component increasing with the amount of saponin required to be used up to bring about lysis, and the amount of that component increasing with the resistance. This would give, of necessity, a positive correlation, whether by rank or by absolute value, and not a negative one.

We have already suggested [Ponder, 1926, 1] that the component with which the saponin interacts is a protein component of the cell wall, and so we may correlate the resistance to saponin with the protein contents of the various cells, as given by Abderhalden, excluding, of course, the content of haemoglobin. This procedure at once gives the high positive coefficient of  $0.79$ . The positive sign, moreover, gives the coefficient a real significance, for we can say that the more protein in the cell, the more saponin requires to be

used up to bring about lysis, and the more resistant is the type of cell accordingly. Or, reversing the argument, the more protein in the cell, the more readily is the cell haemolysed with hypotonic saline.

We suggest that it is the protein content which is the primary factor in the determination of the resistance to saponin and to saline, and that the observation of Port is due to the fact that there exists a coefficient of rank correlation between protein content and phosphoric acid of  $-0.75$ . Why this latter figure should appear we do not know, nor is its appearance material to the question at issue. The suggestion that the protein content is the essential factor at least brings Ryvosh's series into line with the results of previous researches, all of which indicate that the action of saponin is on the protein component of the cell. The phosphoric acid content happens to be a guide to the resistance merely because the amount of this substance varies inversely, roughly speaking, with the protein content.

To express the conclusion concisely, the evidence points to the following state of affairs. In a cell such as that of the sheep, rich in protein, much saponin requires to be transformed by the formation of a compound with this protein before lysis is brought about. The cell is therefore very resistant to saponin. The same cell is readily haemolysed by hypotonic saline, whether because of its high protein content, or for some other reason which we cannot indicate. A cell poor in protein, such as that of the rat, requires little saponin for the transformation of sufficient of the cell wall to bring about haemolysis, and so appears relatively unresistant to saponin. At the same time, it requires a relatively low tonicity to bring about lysis, and the cell is thus relatively resistant to hypotonic saline.

There is no difficulty in appreciating why a large protein content should give a high resistance to saponin. The reason why a high protein content should accompany a low resistance to hypotonic saline is more difficult to discover, and must be sought in the mode of operation of hypotonic saline on the cell. It may be that, just as the resistance to saponin is determined by the protein content but indicated by the phosphoric acid content, so the resistance to hypotonic saline is indicated by the protein content, but determined by some factor which is negatively correlated to the amount of protein in the cell.

#### SUMMARY.

1. The series of Ryvosh is investigated afresh by quantitative methods. Ryvosh's results are in the main confirmed, and his conclusions amplified by the calculation of the correlation coefficients applicable to the series.
2. It is suggested that the resistance of cells to saponin is principally determined by the protein content of the cell, exclusive of haemoglobin, and the evidence for this suggestion is discussed.

This research was carried out under a Grant from the Government Grants Committee of the Royal Society.

## REFERENCES.

- Abderhalden (1895). *Z. physiol. Chem.* 25, 67.  
Brinkmann (1922). *Ned. T. v. Gen.* 66, 2464.  
Orahovats (1926). *J. Physiol.* 61, 447.  
Ponder (1923). *Proc. Roy. Soc. Lond. B*, 95, 42.  
—— (1926, 1). *Proc. Roy. Soc. Lond. B*, 100, 199.  
—— (1926, 2). *Proc. Roy. Soc. Lond. B*, 99, 461.  
—— (1926, 3). *Biochem. J.* 20, 507.  
Port (1922). Höber's *Physikalische Chemie*, p. 593.  
Ryvosh (1907). *Pflüger's Arch.* 116, 229.



## XI. THE BIOCHEMISTRY OF THE AQUEOUS HUMOUR.

By STEWART DUKE-ELDER (*Henry George Plimmer Research Fellow*).

*From the Biochemical Department, St George's Hospital, London.*

(Received December 3rd, 1926.)

THE view at present most generally accepted regarding the origin and nature of the aqueous humour of the eye is that it is secreted by the cells of the ciliary epithelium, that it circulates through the eye, and that it finds an exit therefrom largely by way of the canal of Schlemm. Alternatively it is held by some that this fluid is formed from the blood by a process of simple transudation through the capillary walls and circulates in a similar manner. The evidence whereon the secretory theory is based is largely anatomical, resting on the histological structure of the ciliary epithelium and its resemblance to a gland—a form of evidence at best inconclusive and susceptible of varying interpretations. Some animals possess no ciliary body at all; others, possessing a ciliary body, show no specific glandular formation; and finally, in those animals (*e.g.* man) wherein the anatomical evidence is most complete, the resemblance to true glands is questioned by many authorities. Moreover, even in these latter it can be shown that the formation of the intra-ocular fluids, though largely associated with the ciliary body, is by no means confined to it. The “circulation” of the aqueous humour has been determined and measured only by unsound physiological experiments wherein the normal pressure conditions have been upset by opening the eye, or by making injections into the eye, or by employing other procedures which can be interpreted as calculated to initiate artificially an abnormal circulation.

In a series of experiments on the intra-ocular pressure it was indicated [1926, 1] that the pressure equilibrium of the eye might be explained more consistently on the hypothesis that it was of a hydrostatic rather than of a hydrodynamic nature. These suggested the probability of a third hypothesis which has more recently been put forward, that the aqueous humour is formed by dialysis from the blood, and that, apart from a minimal circulation determined by metabolic interchange and muscular activity, it is in equilibrium with it. A further series of experiments on the vascular pressures of the eye [1926, 3] demonstrated that the physical forces involved in the production of the aqueous humour did not necessitate the intervention of any secretory energy; the difference between the intra-ocular pressure and the hydrostatic pressure in the ocular arteries and veins, and the difference in

osmotic pressure between the aqueous humour and the capillary plasma were found to be compatible with a dialysis hypothesis. It was shown also that the pressure equilibrium forbade a hydrostatic outflow of aqueous humour into the veins under normal pressure conditions [1926, 2], and that, in addition, the differences in osmotic pressure [1927] and electrical potential between the plasma and the aqueous humour were of an order such as to satisfy the requirements of a system in thermodynamical equilibrium.

In complicated physiological experiments the number of imperfectly controllable variables frequently leads to equivocal results and rarely to a pragmatic conclusion; whilst the attempt to eliminate the variables tends to introduce conditions so abnormal as to defeat its own ends. It was therefore felt that the study of the aqueous humour from the biochemical point of view would not be without interest. The main difficulty which presented itself was the small quantity of intra-ocular fluid which is available from any one animal, and the minute concentration of some of the constituents. In order to get a general comparison between the aqueous humour and the blood, large quantities of horse aqueous humour were obtained, and the pooled fluid was analysed concurrently with a typical sample of horse serum. By this procedure a qualitative comparison could be made, and though it cannot lay claim to quantitative accuracy, a relatively close approximation can be obtained. The principal constituents of each class of substance were then selected and micro-estimations made of these, comparing each with the corresponding concentration in the arterial and venous plasma of the same animal. Finally, changes in the chemical constitution of the aqueous humour were studied under experimentally produced variations in the blood and in the eye.

# 1. THE GENERAL CHEMISTRY OF THE AQUEOUS HUMOUR.

A considerable amount of work has been done on the general chemistry of the aqueous humour—in the horse notably by Mestrezat and Magitot [1921]—but in no case has a systematic analysis been directly compared with the blood.

*Collection of material.* The aqueous humour was taken from the eyes of horses immediately after their being slaughtered. In this animal each eye contains 1 to 2 cc. of fluid; only the first cc. was withdrawn, since on complete evacuation the last drawn fluid becomes contaminated with plasma exuded from the capillaries. The cornea was cleaned and dried, a nick made through three-quarters of its substance with a sharp knife, and the needle of a 1 cc. syringe inserted through it, care being taken to avoid the iris and to cause as little pressure disturbance as possible. The serum was a typical sample taken from the farm of the Medical Research Council.

The chemical analysis is given in the accompanying table.

*Methods of analysis.* The total protein was estimated in the aqueous humour by acidifying 100 cc. and coagulating by heat; the precipitate was washed with water and alcohol, dried at 100° and weighed. 10 cc. of serum

Quantities in g. per 100 cc.

				Aqueous humour	Serum
Water	...	...	...	99.6921	93.3238
Solids (dried at 100°)	...	...	...	1.0869	9.5362
Total protein	...	...	...	0.0201	7.3692
Albumin	...	...	...	0.0078	2.9557
Globulin	...	...	...	0.0123	4.4135
Non-protein N	...	...	...	—	0.0239
Total N	...	...	...	0.0268	—
Urea	...	...	...	0.028	0.027
Amino-acids	...	...	...	0.029	0.035
Creatinine	...	...	...	0.002	0.002
"Fats"	...	...	...	Trace (0.004)	0.13
Cholesterol	...	...	...	? Nil	—
"Sugar"	...	...	...	0.0983	0.0910
Sodium	...	...	...	0.2787	0.3351
Potassium	...	...	...	0.0189	0.0201
Calcium	...	...	...	0.0062	0.0101
Magnesium	...	...	...	0.0026	0.0028
Chlorine	...	...	...	0.4371	0.3664
Inorg. P ( $P_2O_5$ )	...	...	...	0.0033	0.0030
Inorg. S ( $SO_4$ )	...	...	...	0.0061	0.0058

diluted to 100 cc. with distilled water were treated similarly. The albumin was determined by precipitating the globulins by adding an equal volume of saturated ammonium sulphate solution to 100 cc. which had been allowed to concentrate in a desiccator, allowing this to stand for 24 hours, and filtering: the filtrate was acidified with *N*/10 sulphuric acid and filtered; the precipitate was redissolved in the original volume of distilled water, neutralised with dilute caustic soda, and re-precipitated with half-saturated ammonium sulphate, the albumin being precipitated again from the filtrate with acid. This process of fractionation was repeated five times. The final filtrate was acidified and boiled, the precipitate washed with water, alcohol, and ether, dried at 100° and weighed. 10 cc. of diluted serum were treated similarly. The globulin was taken as the difference between the total protein and the albumin.

The total nitrogen of the aqueous humour was estimated by a micro-Kjeldahl determination; the non-protein nitrogen of the serum by the same method on a protein-free filtrate (Folin and Wu). Urea was estimated by the urease method: the amino-acids by Van Slyke's method: and the creatinine colorimetrically against a standard creatinine picrate solution.

By the term "fats" is meant the figures obtained by weighing an ether extract of dried residue. The cholesterol was estimated after extraction by precipitation with digitonin.

"Sugar" is taken as reducing substance estimated as glucose by the Schaffer-Hartmann method.

The total mineral ash was obtained by calcination. The material thus left was dissolved in HCl; barium chloride and baryta were added, and the phosphates and sulphates removed; barium salts were removed by ammonia and ammonium carbonate; the filtrate was evaporated to dryness and the ammonium salts removed by heat; the residue was dissolved, treated again with ammonia and ammonium carbonate, filtered, acidified with HCl and

evaporated to dryness. This residue was redissolved; the potassium was estimated by precipitation with  $\text{H}_2\text{PtCl}_6$ , and the amount of sodium calculated by difference. The calcium was precipitated as oxalate, and estimated as  $\text{CaO}$ , the magnesium as ammonium magnesium phosphate and estimated as magnesium pyrophosphate. The chlorides were determined by Ruszynák's modification [1921] of Koranyi's method; the inorganic phosphates gravimetrically as pyrophosphate; and the inorganic sulphates by conversion to benzidine sulphate and titration with sodium hydroxide.

The results of the chemical analysis show that the constituents of the aqueous humour, when compared with those of serum, may be divided into three groups of substances depending on the physical state of their molecules in solution.

1. *Colloidal substances. Partition coefficient serum/aqueous humour*  $> 1$ .

All the colloidal substances are found in the aqueous humour in much smaller concentration than in the serum. Proteins are present in the former in very small quantities; but it is to be noted that although this is the case the different fractions (as determined by precipitation by ammonium sulphate) are found in approximately the same proportion as they occur in the blood. Thus the albumin/globulin ratio in the former is 38.8/61.2, in the latter 39.9/60.1. Further it was shown by dialysing the globulin fraction against distilled water that in the aqueous humour as in the serum it was composed partly of euglobulin and partly of pseudoglobulin, although, unfortunately, the minuteness of the quantities involved precluded any reliable quantitative estimation. Moreover, it can be shown that they are specifically identical<sup>1</sup>. Similarly the "fats," also indiffusible substances, are found in correspondingly minute quantities in the aqueous humour.

Since all the colloidal constituents of the serum appear only in traces in the aqueous humour the two solutions are of very dissimilar molecular aggregation; in comparing the distribution of the diffusible constituents it is therefore necessary to apply a correction factor to allow for the difference in solid displacement due to the unequal mass of solute. Thus 100 cc. of the horse aqueous humour contain 1.0869 g. of solids and 99.6921 g. of water, whilst the same quantity of serum contains 9.5362 g. of solids (largely protein) and 93.3238 g. of water. A correction factor therefore of  $100/99.6921$  or 1.003 applied to the aqueous humour and of  $100/93.3238$  or 1.07 applied to the serum gives comparative results expressed as concentrations dissolved in an equal quantity (100 g.) of water.

2. *Diffusible non-dissociated substances. Partition coefficient*  $= 1$ .

The total N of the aqueous humour was found to be 0.0268 g. %. Deducting 0.0032 g. % to allow for 0.02 % protein, the non-protein nitrogen becomes 0.0236 g. %—a close approximation to the non-protein nitrogen

<sup>1</sup> Unpublished researches, at present being extended, with Dr Percival Hartley.

content of the serum (0.0239 g. %). The amino-acid content at any one time is probably too variable a quantity to permit reliable deductions to be made from it when the method of collecting materials is borne in mind. The creatinine appeared to be present in both in equal quantities. The urea and the "sugar" also appeared in practically the same concentrations.

	Aqueous humour		Serum	
	G. per 100 cc. solution	G. per 100 g. water	G. per 100 cc. solution	G. per 100 g. water
Urea	0.028	0.028	0.027	0.0289
"Sugar"	0.0983	0.0986	0.0910	0.0974

The diffusible non-dissociated substances are therefore partitioned between the two fluids in approximately equal amounts.

### 3. *Dissociated diffusible substances.*

The dissociated substances are seen to be unequally distributed, even when corrected for solid displacement. In each case the cations have a partition coefficient greater than one, and the anions a partition coefficient less than one.

	Aqueous humour			Serum		
	Per 100 cc. solution		Per 100 g. water g.	Per 100 cc. solution		Per 100 g. water g.
	g.	Milli-mols per litre		g.	Milli-mols per litre	
<i>Cations:</i>						
Sodium	0.2787	121.2	0.2795	0.3351	145.6	0.3585
Potassium	0.0189	4.8	0.0190	0.0201	5.1	0.0215
Calcium	0.0062	1.5	0.0063	0.0101	2.5	0.0108
Magnesium	0.0026	1.1	0.0026	0.0928	1.2	0.0030
<i>Anions:</i>						
Cl'	0.4371	123.1	0.4384	0.3664	103.2	0.3920
PO <sub>4</sub> '''	0.0044	1.38	0.0044	0.0040	1.26	0.0043
SO <sub>4</sub> ''	0.0061	1.8	0.0062	0.0058	1.7	0.0062

It is seen that in the serum there is a considerable excess of basic radicles, which is shown especially in the case of sodium and calcium. This is accounted for by the fact that in blood part of these are associated with protein as protein salts, and as such are rendered indiffusible. Rona and György [1913] found that 15 to 28 % of the total quantity of sodium was indiffusible for this reason, and Rona and Takahashi [1913] that 30 to 40 % of the total calcium was similarly in association with protein. Conversely, the acidic radicles show a predominance in the aqueous humour, the chlorides showing a higher relative concentration than the phosphates and sulphates. This again suggests comparison with the work of previous observers, since all the chlorides of serum have been found to dialyse [Ascher and Rosenfeld, 1907; Creveld, 1921], while the phosphates have been held back to some extent in loose combination with the protein. The increase in the concentration of anions as a whole and the diminution of cations strongly suggest that the distribution of the ionic activities between the aqueous humour and the blood is subject to the

thermodynamical laws relating to two fluids in the membrane equilibrium described by Donnan. Thus the relative concentrations expressed as normal chlorine and sodium are:

$$\text{Cl}_{\text{aq}} : \text{Cl}_{\text{serum}} = 123 : 103$$

$$\text{Na}_{\text{aq}} : \text{Na}_{\text{serum}} = 121 : 145.$$

The theoretical relation characteristic of such an equilibrium

$$[\text{Na}^+]_{\text{aq}} \times [\text{Cl}^-]_{\text{aq}} = [\text{Na}^+]_{\text{serum}} \times [\text{Cl}^-]_{\text{serum}}$$

therefore becomes

$$121 \times 123 = 145 \times 103$$

or

$$148.83 = 149.35.$$

The distribution of all the constituents of the aqueous humour thus appears to be determined by physical laws and provides no evidence of the expenditure of any secretory energy in its elaboration, whilst the concentration of its ionised substances seems to preclude a simple transudation, but rather suggests that it is formed by dialysis from the blood through a membrane which is almost impermeable to colloidal micelles and is in equilibrium with it.

## II. THE "SUGAR" AND SALT OF THE AQUEOUS HUMOUR.

In order to compare the aqueous humour with the blood under more exact conditions than were possible in the method of collection of material detailed above, two of the most abundant and easily manipulated constituents were chosen—"sugar" as being representative of the non-dissociated substances, and chlorides as being representative of the ionised constituents—and their relative concentrations compared. The two fluids—aqueous humour and plasma—were in each case derived from the same animal and were retained in a condition approximating the normal as closely as possible.

Rabbits were used. The aqueous humour was withdrawn under sterile conditions by means of a syringe dried with alcohol and ether. The needle had a broad lance point which was introduced into the cornea obliquely near the limbus; such a needle is inserted with less disturbance than the ordinary round pointed instrument, and with the latter it was found difficult to prevent aqueous humour escaping round it at the moment of introduction. Cocaine (2 %) was instilled into the conjunctival sac as an anaesthetic; it has been repeatedly demonstrated that this procedure does not appreciably alter the properties of the intra-ocular fluids. Before the needle was introduced the cornea was dried with blotting paper to obviate any contamination with lacrymal secretion which is of widely different composition. Blood was taken also with aseptic precautions from the ear, the central artery or the marginal vein being used as the case required. Plasma in preference to serum was employed, since it is the former which comes into equilibrium with the aqueous humour *in vivo*; the use of anti-coagulants was dispensed with, since these substances are known to alter the distribution of its constituents to such an extent as to make estimations carried out *in vitro* useless for comparative

purposes with the actual state of the blood *in vivo*. Blood was therefore sucked directly through a paraffined needle and tube into a paraffined centrifuge tube under a layer of paraffin, and, after centrifuging, the middle layer of plasma was pipetted off without its ever having been in contact with air. The centrifuging was done rapidly, and in this way both arterial and venous plasma were obtained in a state as near to that in which they occur naturally as experimental manipulations permitted.

The results are expressed in g. per 100 cc. solution, and g. per 100 g. water. 100 cc. rabbit's plasma were found to contain 8.6832 g. solids (dried at 100°), and the density was 1.023. Correspondingly, 100 cc. aqueous humour contained 1.0899 g. solids (dried at 100°), and its density was 1.007. The factors 1.07 and 1.003 respectively therefore give the concentration of solution in 100 g. water.

The "sugar" (*i.e.* reducing substance) was estimated by the Hagedorn-Jensen method.

	No. of rabbit	G. per 100 cc. solution	G. per 100 g. water
Aqueous humour	1	0.141	0.141
	2	0.138	0.139
	3	0.175	0.175
Arterial plasma	1	0.136	0.145
	2	0.139	0.148
	3	0.170	0.182
Venous plasma	1	0.120	0.128
	2	0.111	0.118
	3	0.143	0.153
Average aqueous humour		0.151	0.151
" arterial plasma		0.148	0.158
" venous plasma		0.125	0.133

The sugar concentration of the aqueous humour lies between that of arterial and venous plasma, a relation which suggests that it comes into equilibrium with capillary plasma. The concentration appears to be more closely related to the arterial than to the venous plasma; this finding may be compared with that of Foster [1923], who showed that the sugar content of "finger blood," and therefore presumably of capillary blood, was very nearly identical with that of arterial and widely different from that of venous blood. The results obtained may be compared with those of previous observers on the sugar content of aqueous humour. Osborne [1919] found the concentration in both to be the same; Ask [1914] found the sugar in the aqueous humour to be 0.01 to 0.02 % greater than in the blood. Deiter [1925] 0.004 % greater; whilst Haan and Creveld [1921, 1, 2] obtained a value for the aqueous humour 0.045 % less than that for the blood. These last authors accept the hypothesis of the dialysis of the aqueous humour, and, taking the sugar content of the capillary plasma as being the mean of the arterial and venous plasma and finding the concentration in the aqueous humour less than this, they conclude therefrom that glucose is partially retained in combination with plasma proteins. Until more exact knowledge of the sugar content of capillary

blood is available it would seem dangerous to draw any such conclusions from the above figures. The results obtained in the present investigation suggest, however, that the sugar content of the aqueous humour lies between that of the arterial and venous plasma and support the hypothesis of the dialysis of the aqueous humour.

*Salt.* The chloride was estimated by Ruszynák's modification of Koranyi's method, and is expressed as NaCl.

	No. of rabbit	G. per 100 cc. solution	G. per 100 g. water
Aqueous humour	1	0.668	0.670
	2	0.597	0.599
	3	0.641	0.643
Arterial plasma	1	0.603	0.645
	2	0.543	0.581
	3	0.590	0.631
Venous plasma	1	0.578	0.618
	2	0.501	0.536
	3	0.573	0.613
Average aqueous humour		0.635	0.637
„ arterial plasma		0.579	0.619
„ venous plasma		0.551	0.589

The chloride content of the aqueous humour is higher than that of the plasma even when expressed as g. in an equal weight of water. The results obtained thus agree with those of Ascher [1922] and Creveld [1921]—and again suggest that the aqueous humour is a dialysate from the blood.

### III. THE CHEMICAL COMPOSITION OF ABNORMAL AQUEOUS HUMOUR.

If the intra-ocular fluids are a dialysate of the plasma there are two possible methods of changing their composition: by altering the permeability of the dialysing membrane (*i.e.* the capillary wall), and by altering the composition of the blood.

#### 1. *Alteration of the permeability of the capillary walls.*

In a state of dilatation the permeability of the capillary walls is increased, and under these circumstances a larger proportion of colloidal molecules is able to penetrate through their walls. This occurs in the eye as elsewhere, and, along with the increased protein content of the aqueous humour, definite changes in the distribution of ionised substances occur.

*(a) Dilatation of the capillaries by paracentesis.* When the eye is punctured and the intra-ocular fluids are withdrawn the capillaries, deprived of the supporting pressure of the aqueous humour, undergo immediate dilatation. The fluid re-formed under these conditions I have elsewhere [1927] called "plasmoid aqueous." The aqueous humour was withdrawn from the eyes of rabbits, using the same technique as was employed previously, and the re-formed fluid was similarly withdrawn 20 minutes after the first paracentesis.

The chemical constitution of the normal and plasmoid aqueous humours was compared in respect of their colloid, sugar, and chloride contents.



*Colloid content.* The colloids, which are largely composed of protein, were estimated refractometrically. A dipping refractometer (Zeiss) was used provided with an auxiliary prism to enable it to deal with one drop of fluid and the temperature was kept constant by a thermostat; the error of the instrument is  $\pm 3.7$  units of the fifth decimal place of  $n_D$ .

The increase of refractivity found in the "plasmoid aqueous" shows an increase in its content of colloids (see table below)—a fact which has long been known. Considering the total colloid as proteins, the approximate corresponding percentages of this substance are also given. The figures do not pretend to any great accuracy. They were calculated by the technique suggested by Robertson [1915] applied to a large quantity (100 cc.) of horse aqueous humour. The refractive index of a protein-free preparation of this fluid was subtracted from that of the normal horse aqueous humour, and the differential result thus obtained was correlated with a gravimetric estimation of the total protein in the original fluid. That this refractive index was due to increase of protein, both albumin and globulin, was seen by special tests for these substances. The Noguchi globulin test gave a granular flocculent precipitate whilst Pandey's reaction gave a typical blue-white cloud; both these reactions are absent from the normal aqueous humour. Similarly the Nonne-Apelt test gave a positive reaction for globulin, and on filtering and acidifying the filtrate the presence of increased albumin was verified.

						Refractive index
Normal horse aqueous humour	...	...	...	...	...	1.335130
Protein-free horse aqueous humour	...	...	...	...	...	1.335091
Refractive index of protein	...	...	...	...	...	0.000039
Protein	...	...	...	...	...	0.024 %

The aqueous humour of the rabbit was found to have on the average a higher refractive index than that of the horse.

						Refractive index
Normal rabbit aqueous humour	...	...	...	...	...	1.335168
Protein-free rabbit aqueous humour	...	...	...	...	...	1.335091
Refractive index of protein	...	...	...	...	...	0.000077
Protein	...	...	...	...	...	0.04 % (approx.)

No. of rabbit	Aqueous removed cc.	$n_D$ Normal aqueous	$n_D$ "Plasmoid aqueous"	Difference (plasmoid - normal)	Approx. % protein
1	0.1	1.335244	1.337088	0.001844	1.0
2	0.2	1.335130	1.338428	0.003298	1.8
3	0.25	1.335168	1.339036	0.003868	2.0
4	0.32	1.335244	1.339834	0.004590	2.5

Concurrent sugar and salt estimations showed the following results. The sugar was estimated by the Hagedorn-Jensen method and is expressed as g. per 100 cc.

No. of rabbit	Normal aqueous	"Plasmoid aqueous"	Difference
1	0.143	0.148	0.005
2	0.155	0.173	0.018
3	0.173	0.175	0.002
4	0.165	0.172	0.007

The increase of sugar in the "plasmoid aqueous" is probably largely to be explained by an increase in the blood sugar occurring during the experiment due to emotional excitement on the part of the animals. An effort was made to accustom the rabbits to experimental procedures before performing any of the above experiments upon them; but without success. Thus in one experiment the blood sugar at the start was 0.154 %, and at the end had risen to 0.177 %.

The salt was estimated by the Ruszynák method and is expressed as g. NaCl per 100 cc.

No. of rabbit	Normal aqueous	"Plasmoid aqueous"	Difference
1	0.641	0.600	0.041
2	0.597	0.561	0.036
3	0.680	0.536	0.144
4	0.500	0.421	0.079

There is therefore a constant decrease in the quantity of chloride with increasing concentration of protein.

(b) *Dilatation of the capillaries by radiant energy.*

(i) *Ultra-violet rays.* Rabbit—10 minutes' irradiation at 2 feet from a quartz mercury vapour lamp, water-cooled. The left eye protected.

Rabbit	...	...	Left eye normal	Right eye irradiated
Refractive index			1.335168	1.340898 (approx. 3 % protein)
"Sugar"			0.154	0.172
Chloride			0.693	0.520

(ii) *Infra-red rays.* Rabbit—10 minutes' irradiation at 2 feet from a carbon arc, ultra-violet filtered off through 1 cm. thickness of glass. The left eye protected.

Rabbit	...	...	Left eye normal	Right eye irradiated
Refractive index			1.335247	1.341990 (approx. 3.5 % protein)
"Sugar"			0.125	0.131
Chloride			0.661	0.501

It is probable that both of these agencies act in the same way, the radiant energy being absorbed by the pigment of the iris, and there converted into heat which causes capillary dilatation.

(c) To show that the changed chemical constitution was due to the dilatation of the capillary walls, a paracentesis was done after 1 cc. of 1 : 1000 adrenaline with cocaine had been injected behind the eyeball. This counteracts to a large extent the dilatation caused by the paracentesis. Wessely [1908] first showed that the aqueous humour formed secondarily under these conditions contained less protein than the usual "plasmoid aqueous": the present analysis demonstrates that throughout it is to a less extent of a plasmoid nature, but resembles more closely the normal fluid.

Rabbit	...	...	Left eye—normal	Right eye—paracentesis after adrenaline
Refractive index			1.335130	1.335244
"Sugar"			0.152	0.175
Chloride			0.638	0.630

It is suggested that whilst the normal aqueous humour has the chemical constitution which one would expect were it in membrane equilibrium with the capillary plasma, the abnormal humour formed under experimentally varied conditions changes its composition in a corresponding manner. On increasing the permeability of the separating membrane and allowing more colloid to enter the eye, the increase is proportional to the amount of capillary dilatation produced. With the tendency to equalisation of the amount of colloid on either side of the membrane the excess of cations largely disappears, while, allowing for the increased glucose content of the blood which accompanies the excitement of the experimental manipulations, the non-dissociated constituents ("sugar") remain practically constant. Moreover, on preventing capillary dilatation the aqueous humour formed secondarily after puncture of the eye is not far removed from the normal. It would appear, therefore, that the view is unjustified which ascribes to the normal humour and the "plasmoid aqueous" fundamentally different origins—that the former is a secretion and the latter a transudation; it would seem rather that the intra-ocular fluids are formed under both conditions by the same process of dialysation through a membrane of varying permeability.

## 2. *By varying the chemical constitution of the blood.*

A series of experiments was done on cats wherein varying quantities up to 50 cc. of a 10 % solution of gum arabic were injected intravenously: no change in the refractive index of the aqueous humour was ever detected. Where colloidal substances are susceptible to delicate biological tests, however, their presence can be detected in proportions corresponding to the traces of protein normally present, as is seen in the case of the various substances associated with immunity [Poleff, 1914, and others]. Difficultly diffusible drugs are also found in traces—*e.g.* the organic compounds of arsenic [Neame and Webster, 1923]. All these substances are found in the "plasmoid aqueous" in quantity.

Diffusible substances find their way freely into the aqueous humour. Thus in experiments carried out on the cat [1926, 1] when 10 cc. of a 30 % glucose solution were injected intravenously, the sugar content of the right eye before the experiment was found to be 0.146 %, whilst that in the left 15 minutes after the injection was 0.204 %. Similarly with sodium chloride; the concentration of this substance was found to rise from 0.641 to 0.698 % after the intravenous injection of 10 cc. of a 30 % solution. This corresponds with the clinical findings of Ask [1914] and Deiter [1925], who found that the sugar content of the aqueous humour rose parallel with the blood in diabetics, and of Gala [1924], who found that in cases of chloride retention the aqueous humour varied similarly, and the experimental findings of Löhlein [1910] and Haan and Creveld [1921, 2], who found that such substances as fluorescein, potassium ferrocyanide, and potassium iodide were partitioned between the aqueous humour and the blood in the ratio of their diffusion constants.

SUMMARY.

From the biochemical point of view neither the normal nor the abnormal aqueous humour shows evidence of the existence of any special secretory mechanism in its elaboration but appears to be rather a dialysate of the capillary blood.

I am indebted to the Research Committee of St George's Hospital Medical School for financial assistance in the Sir Francis Laking Prize; and to Mr J. A. Gardner for advice and criticism.

REFERENCES.

- Ascher (1922). *Arch. Ophthalm.* **167**, 247.  
 Ascher and Rosenfeld (1907). *Biochem. Z.* **3**, 335.  
 Ask (1914). *Biochem. Z.* **59**, 1.  
 Creveld (1921). *Biochem. Z.* **123**, 304.  
 Deiter (1925). *Arch. Augenheilk.* **96**, 179.  
 Duke-Elder (1926, 1). *Brit. J. Ophthalm.* **10**, 1.  
 ——— (1926, 2). *J. Physiol.* **61**, 409.  
 ——— (1926, 3). *J. Physiol.* **62**, 1; *Brit. J. Ophthalm.* **10**, 513  
 ——— (1927). *J. Physiol.* In the Press.  
 Foster (1923). *J. Biol. Chem.* **55**, 291.  
 Gala (1924). *Bratis. Lek. Listy*, **5**, 42.  
 Haan and Creveld (1921, 1). *Biochem. Z.* **123**, 190.  
 ——— (1921, 2). *Biochem. Z.* **124**, 172.  
 Löhlein (1910). *Arch. Augenheilk.* **65**, 318, 417.  
 Mestrezat and Magitot (1921). *Compt. Rend. Soc. Biol.* **84**, 185.  
 Neame and Webster (1923). *Trans. Ophthalm. Soc., U.K.* **43**, 381.  
 Osborne (1919). *J. Physiol.* **52**, 347.  
 Poleff (1914). *Klin. Monat. Augenheilk.* **52**, 819.  
 Robertson (1915). *J. Biol. Chem.* **22**, 233.  
 Rona and György (1913). *Biochem. Z.* **48**, 278.  
 Rona and Takahashi (1913). *Biochem. Z.* **49**, 370.  
 Ruzsnyák (1921). *Biochem. Z.* **114**, 23.  
 Wessely (1908). *Arch. Augenheilk.* **60**, 1, 97.

## XII. THE ABSORPTION SPECTRUM OF CHOLESTEROL AND ITS BIOLOGICAL SIGNIFICANCE WITH REFERENCE TO VITAMIN D. PART I.

### PRELIMINARY OBSERVATIONS.

BY ISIDOR MORRIS HEILBRON, EDWARD DAVID KAMM  
AND RICHARD ALAN MORTON.

*From the Department of Organic Chemistry, University of Liverpool.*

*(Received December 6th, 1926.)*

It has been fully proved by various investigators that although cholesterol itself exhibits no antirachitic properties prior to irradiation with ultra-violet light, these are developed on comparatively short exposure to these rays. This brings the reaction within the sphere of photochemistry and since photochemical changes do not occur without the absorption of light and since it follows from the Grotthus-Draper law that only the rays absorbed are active, it would immediately become a matter of urgency to ascertain whether cholesterol showed well-defined absorption bands in the ultra-violet. The hitherto published results on this aspect of the problem are essentially preliminary in character. Hess and Weinstock [1925] observed that when cholesterol was irradiated by ultra-violet rays a change in the absorption spectrum occurred. The activated material absorbed light of certain wave-lengths to a less degree than ordinary cholesterol, a difference in the absorption over the entire range of wave-lengths (integrated) being detected by the use of a thermopile and galvanometer set. Schlutz and Ziegler [1926], carrying the matter further, observed that carefully recrystallised cholesterol, melting at  $148.5^{\circ}$  or very near that point, showed selective absorption "of wave-lengths between  $294\text{--}296\text{ }\mu\mu$  and  $279\text{--}294\text{ }\mu\mu$  with a great deal of general absorption beyond  $294\text{ }\mu\mu$ ." The absorption bands, which were evidently very shallow, could not be detected in alcoholic solution but showed up in ether or chloroform. Moreover, the bands were only observed in the first crop (five fractions collected) from a fractional crystallisation from alcohol of cholesterol (M.P.  $148.5^{\circ}$ ) which had previously been recrystallised seven times from the same solvent.

As these results are not wholly consistent with the idea of ready photochemical change in the cholesterol molecule, we deemed it advisable to undertake a still more extensive and quantitative study of the absorption spectrum of this compound.

The results now to be described were obtained using a Hilger quartz spectrograph, rotating sector-photometer, iron-nickel arc arrangement. The arc (5 amps., 105 volts) was situated at a distance of 132 cm. from the absorbing solution. In our first experiments a sample of carefully purified cholesterol (from brain), M.P.  $148.5^{\circ}$ , kindly supplied by Prof. Drummond,

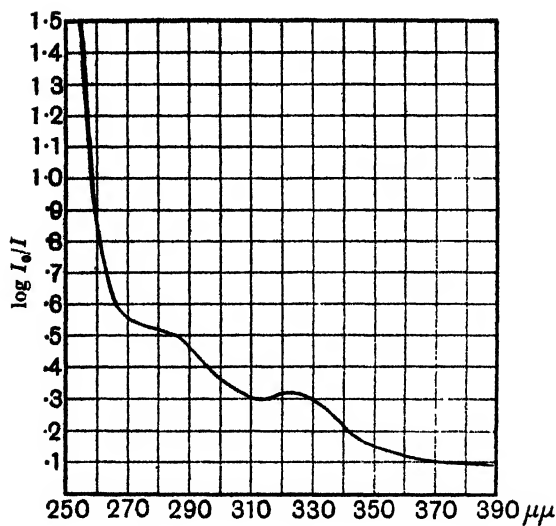


Fig. 1. Cod-liver oil film approx.  $\frac{1}{16}$  mm. thickness.

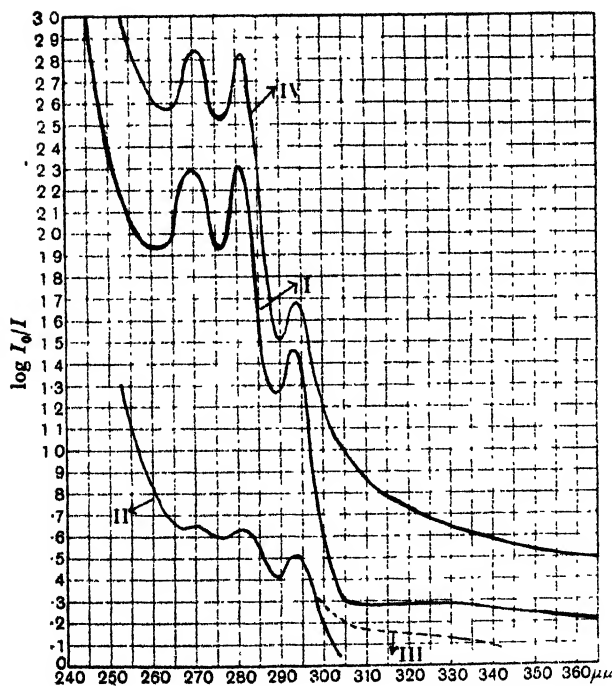


Fig. 2. I. Cholesterol ( $2\frac{1}{2}$  g. from 200) 2 g. in 50 cc. ether, 4 cm. cell, fresh.  
 II. Drummond's cholesterol from brain—our ordinary purified  $148.5^\circ$  cholesterol.  
 III. Solution II after taking 1 plate (i.e. after standing 1 hour).  
 IV. Cholesterol ( $2\frac{1}{2}$  g. from 200) 2 g. in alcohol, 4 cm. cell, fresh.

was employed and showed clearly the very shallow bands recorded by Schlutz and Ziegler [1926]. In addition it gave indications of an extended region of absorption on the ultra-violet side (Fig. 2, curve II). We next employed a sample of cholesterol obtained from cod-liver oil (M.P.  $148.5^{\circ}$ ) and recrystallised by us three times. The absorption curve coincided exactly with that obtained in the previous case. An examination of the curves seemed to us to indicate that the observed curve was made up of two separate curves, one of which we regard as being due to cholesterol itself and the other to the material which becomes active after absorbing light energy.

With the idea of developing and testing this point of view, the unused portion of this cholesterol was once more recrystallised from ethyl acetate, the least soluble 2 g. being again examined spectrographically. It was found that this fraction gave a higher extinction, a higher definition for the bands and made the existence of a third band near  $269\text{ }\mu\mu$  certain (Fig. 3, curve III).

As these preliminary results indicated the presence of some foreign material in cholesterol to which the selective absorption could be attributed, we next undertook fractional crystallisation of 200 g. of our crude material. After one recrystallisation from ethyl acetate (1500 cc.) 50 g. were obtained, M.P.  $147.5\text{--}148^{\circ}$  (Charlottenberg certificated thermometer), and this portion was again dissolved in the same volume of ethyl acetate and the solution allowed to crystallise slowly. The portion which crystallised out (25 g.) melted sharply at  $148.5^{\circ}$ , the melting point being, if anything, fractionally higher than that of the pure specimen of brain cholesterol obtained from Prof. Drummond, which was at the same time re-determined as a check. Two further crystallisations of this material were carried out:

(a) the 25 g. were crystallised from 600 cc. ethyl acetate, yielding 5 g. of a less soluble portion;

(b) this latter portion was again dissolved in ethyl acetate (120 cc.), yielding after cooling 2.5 g. of material melting at  $148.5^{\circ}$ .

It will be noted that as the fractional crystallisation proceeded, the solubility of each successive fraction of residual substance decreased to a marked degree and that whereas the crude product was soluble to an extent of 10 % in ethyl acetate at room temperature, the final fraction which represents approximately 1 % of the original material was only soluble in this solvent under the same conditions to an extent of 2 %. It is thus clear that a substance differing in solubility from ordinary cholesterol may be concentrated by fractional crystallisation, but these results are only attained if an adequate quantity of material is originally taken.

A spectrographic examination of this highly concentrated product now showed that in this material the extinction coefficient had increased to approximately four times that of the purest material previously obtained<sup>1</sup> and

<sup>1</sup> We desire to record our thanks to Dr O. Rosenheim and Mr T. A. Webster who have now tested biologically this preparation and report that it is three to four times as active as a sample of the non-concentrated product. This shows remarkable agreement with the spectrographic results. [February 2, 1927. I. M. H.]

that the definition of all three bands was very much improved (Fig. 2, curves I, IV). Consistent results were obtained when the spectrograms were taken in either alcohol or ether solution.

With the idea of still further concentrating our "X-material," a fresh crystallisation was carried out. In this case we started from 2000 g. of the crude cholesterol, obtaining after one crystallisation from ethyl acetate 450 g. of less soluble material (m.p. 147.5–148°). This was repeatedly crystallised from large volumes of ethyl acetate yielding successively the following less soluble fractions: 117 g., 43 g., 6 g. This last portion was further crystallised from dilute alcohol, yielding 5 g. of material the absorption curve of which is reproduced on Fig. 5, curve I. The extinction coefficient has again increased beyond the highest point previously recorded, but, on the other hand, the bands, rather contrary to our expectation, have lost somewhat in definition.

An important fact regarding this highly concentrated material is that its melting point is not only definitely lower than that of purified material isolated from the less concentrated portions, but is indefinite over a range 146.5–149°. This result is quite consistent with our assumption of an "X-compound" in cholesterol. Moreover, this substance is almost entirely accumulated in the least soluble portion, for, on working up the filtrate from which the 43 g. portion was crystallised, a product was obtained of definitely higher melting point (148.3°) which nevertheless only showed very faint selective absorption, while the material (m.p. 148.5°) obtained by concentration of the filtrate from the previous crystallisation (117 g.) followed by careful fractionation showed no selective absorption whatsoever (Fig. 5, curve II).

A full chemical investigation of this least soluble fraction has just been started. While still in the early stages and awaiting confirmation, the results so far obtained certainly indicate the presence of a substance which has a slightly higher carbon content, is not precipitated with digitonin and does not show the typical Liebermann reaction for cholesterol.

It is of some interest to record that the addition of sodium ethoxide to an alcoholic solution of cholesterol (brain cholesterol from Prof. Drummond) favours increased definition to the band at 269  $\mu\mu$  at the expense of the other bands, whereas addition of hydrogen chloride causes the appearance of a new band at 318  $\mu\mu$ . The latter band is not far removed from the second band of cod-liver oil first recorded by Schlutz and Ziegler [1926] and now confirmed by ourselves (Fig. 1). The significance of these results is not at the moment ripe for discussion.

#### *Irradiation of cholesterol.*

When cholesterol is irradiated in the solid state between quartz plates for 10 minutes at a distance of 6 inches from a quartz mercury lamp which had been in use for some time, almost complete disappearance of selective absorption is noted (Fig. 4, curve III). A sample of the brain cholesterol irradiated by Prof. Drummond in an atmosphere of nitrogen gave a smooth curve



showing only general absorption (Fig. 3, curve I). Moreover, all samples of cholesterol irradiated for periods longer than 10 minutes in the solid state or in alcoholic or ethereal solution (Fig. 3, curve II) resulted in the formation of a product exhibiting no signs of selective absorption. In the case of the least soluble fraction obtained from the 2000 g. fractionation, the absorption spectrum after irradiation (Fig. 5, curve III) although showing no bands has nevertheless a very high extinction coefficient.

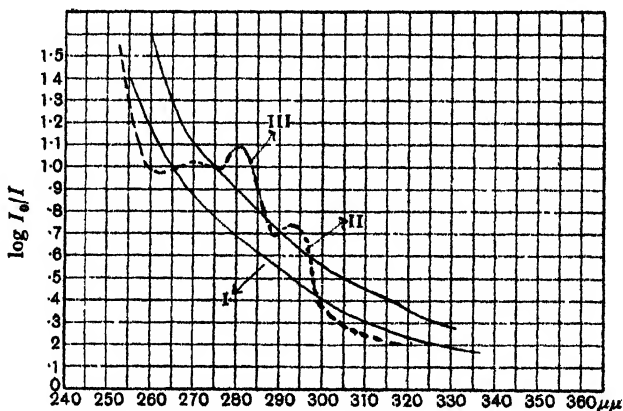


Fig. 3. I Drummond's irradiated cholesterol 2 g. in 50 cc. ether solution, 4 cm. cell.  
II. Cholesterol irradiated in ethereal solution 2 g., 50 cc., 4 cm. cell.  
III. Ordinary 148-5° cholesterol (from cod-liver oil) before irradiation.

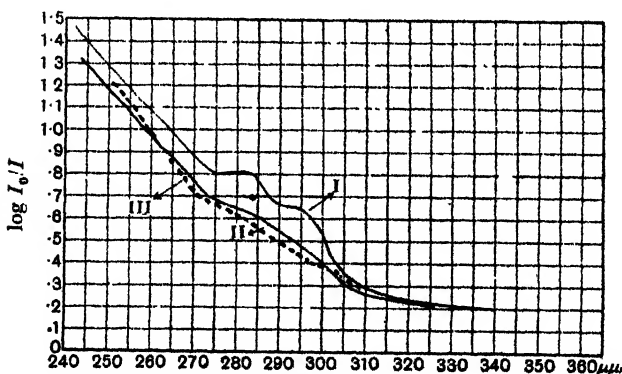


Fig. 4. I. 4 g. ordinary 148-5° cholesterol in 250 cc. alcoholic solution, 10 cm. cell, fresh.  
II. Ditto after 2 hours.  
III. Same cholesterol after 10 minutes' irradiation in solid state.

Finally we have found it impossible again to observe any of the characteristic bands in irradiated material which has subsequently been recrystallised. We have also confirmed the observation of Schlutz and Ziegler [1926] that a solution of cholesterol in alcohol or ether after standing four hours no longer showed the bands (Fig. 4, curves I and II).

DISCUSSION.

The facts that purified cholesterol exhibits selective absorption before irradiation, and that the bands gain in definition and show increased extinction when the material is fractionally crystallised, all point to the presence of an "X-substance" in the original compound. Since the bands disappear on irradiation, with concomitant appearance of vitamin potency, it would seem that there is definite evidence for a correlation between absorption bands and a vitamin precursor. Various lines of investigation combine to show that the precursor is different from cholesterol itself. In the first place, the quantity of antirachitic material produced does not increase beyond a definite limit by prolonging the period of irradiation. This would not be expected if the cholesterol itself were being transformed.

Secondly, as already mentioned, cholesterol recovered from irradiated material no longer shows selective absorption.

Thirdly, Hess, Weinstock and Sherman [1925] failed to prove that deactivated cholesterol could acquire antirachitic potency by renewed irradiation.

Cholesterol becomes active when irradiated in solution—only in those solvents transparent to ultra-violet radiation of the wave-lengths covered by the bands. In acetone (Fig. 6, curve *d*), which effectively absorbs these rays, no activation should occur. This inference is strikingly confirmed by the fact recorded without comment by Hess, Weinstock and Sherman [1925], that irradiation in acetone confers no antirachitic potency.

Two possibilities must not be neglected in interpreting these results: firstly, it is quite conceivable that a material very rich in the vitamin precursor should undergo photochemical change very readily, even to the point of militating against its detection by spectrographic methods. Secondly, cholesterol itself may consist of a mixture of inactive stereoisomerides (compare Anderson and co-workers [1926] on sitosterol) which might differ in absorption spectra. Stereoisomerides frequently exhibit parallel curves differing only in extinction. Either of these alternatives would account for the apparent loss of definition in the bands shown by the end-fraction of the most concentrated material (Fig. 5, curve I).

We must accept, therefore, that cholesterol itself shows only general absorption and that in those fractions which exhibit selective absorption another substance is present. Fig. 6 is a purely geometrical illustration of the type of curve which would result in such a case. It will be seen (*a*) that the resulting summation curve is not dissimilar to the curves actually obtained in our concentrated cholesterol; (*b*) that the ultra-violet component of the band showing a triplet structure necessarily appears in a less well-defined manner than the longer wave component.

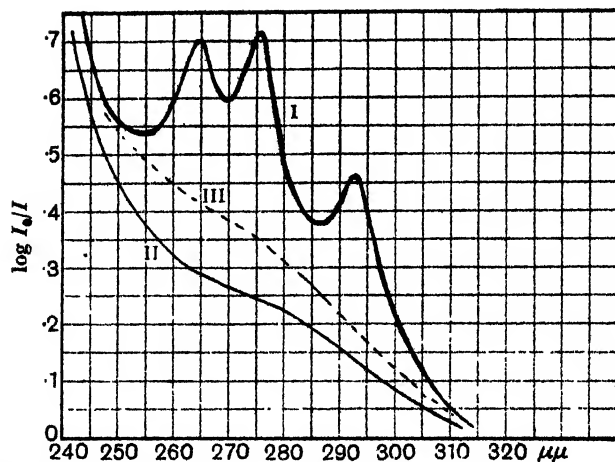


Fig. 5. I. Least soluble fraction from cholesterol.  
 II. Cholesterol after removal of active compound.  
 III. Irradiated fraction.

These curves are not strictly comparable with Fig. 1 but curve I in this figure corresponds with greater absorption than curve IV, Fig. 2.

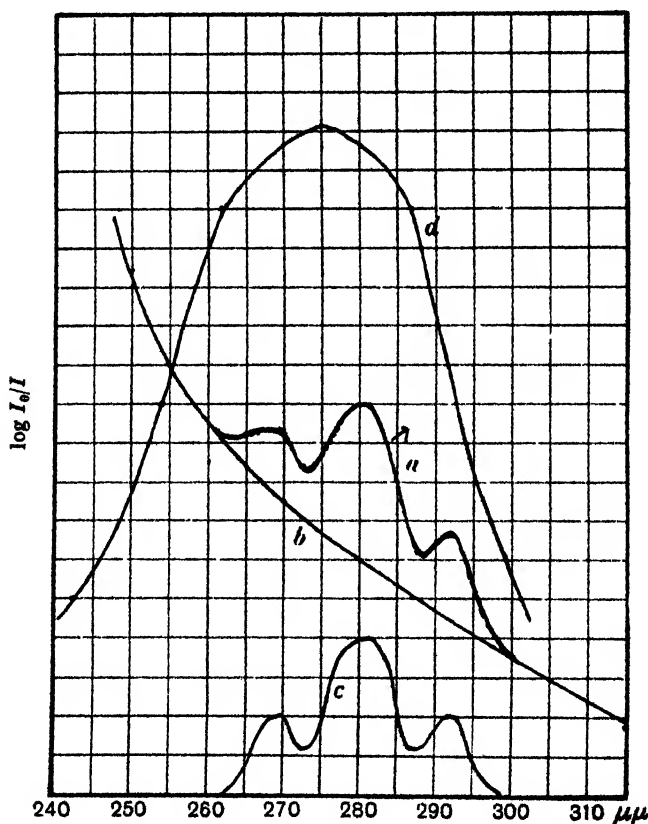


Fig. 6. (a) is a purely geometrical illustration of the effect of superposing selective absorption (c) on general absorption (b). (d) shows the absorption band of acetone on an arbitrary scale.

SUMMARY.

1. Ordinary purified cholesterol contains another compound in small quantity which can be accumulated in the least-soluble fraction.
2. This substance shows well-defined absorption bands at 293  $\mu\mu$ , 280  $\mu\mu$ , and 269  $\mu\mu$ , while cholesterol itself has only general absorption.
3. These bands disappear on irradiation with ultra-violet light with concomitant appearance of antirachitic potency.
4. It is obvious that the unknown substance is closely connected with the vitamin D precursor.

We desire to express our thanks to Prof. J. C. Drummond and Mr H. J. Channon, of the Biochemical Department of University College, London, for providing us with brain cholesterol, to Messrs. Joseph Nathan and Co., Ltd. (Proprietors of Glaxo), for the large quantities of cod-liver oil cholesterol necessary for these experiments, and to the Food Investigation Board of the Department of Scientific and Industrial Research for a grant which has enabled the work to be carried out.

REFERENCES.

- Anderson and co-workers (1926). *J. Amer. Chem. Soc.* **48**, 2972, 2976, 2987.  
 Hess and Weinstock (1925). *J. Biol. Chem.* **64**, 193.  
 Hess, Weinstock and Sherman (1925). *J. Biol. Chem.* **66**, 145.  
 Schlutz and Ziegler (1926). *J. Biol. Chem.* **69**, 415.

[Note added February 2, 1927.] R. Pohl (*Nachrichten der Gesellschaft der Wissenschaften zu Göttingen, Mathematisch-Physikalische Klasse*, 1926) communicated on December 10, 1926, a paper on the absorption spectrum of antirachitic cholesterol. A monochromator with double spectral resolution was employed in conjunction with a photoelectric photometer. The presence of three absorption bands which disappear on irradiation is confirmed and the absorption curve for inactive cholesterol is found to be quite smooth. The photoelectric method, using monochromatic light, thus establishes our view that no change occurs during the measurement of absorption spectra by the photographic method, and our results are fully confirmed.

### **XIII. THE FEEDING OF XANTHOPHYLL TO RATS ON A DIET DEFICIENT IN VITAMIN A.**

BY STANLEY GORDON WILLIMOTT AND THOMAS MOORE.

*From the Biochemical Laboratory, Cambridge.*

*(Received December 6th, 1926.)*

ALTHOUGH the theory of the identity of vitamin A with one of the yellow plant pigments has been abandoned [Drummond and Coward, 1920], most of the experimental work leading to this conclusion has been based upon carotene, while xanthophyll appears to have received less direct attention. This is somewhat surprising in view of the fact that xanthophyll is present in plant tissues in excess of carotene, but is probably accounted for by the well-known fact that vitamin A accompanies carotene rather than xanthophyll in the phase test. Drummond [1919] fed crystalline carotene to rats in the proportion of 0.003 % of the total diet and found it to be ineffective as a source of vitamin A.

Stephenson [1920] removed the carotene of butter fat by adsorption with charcoal without affecting its content of vitamin A.

The evidence is not so complete in the case of xanthophyll. Rosenheim and Drummond [1920] studied the effect of a xanthophyll preparation on rats, but as this material was impure and an indication of growth was obtained, the results were rightly considered inconclusive. On the other hand, Palmer and Kempster [1919] were able to remove xanthophyll from the diet of growing chicks without adversely affecting their health, powers of reproduction, or the virility of their offspring through two generations. The tissues of these birds and their eggs were devoid of carotene and xanthophyll.

Signs of returning interest in the carotenoid pigments are indicated in the paper of Drummond, Channon and Coward [1925]. These authors found that vitamin concentrates prepared from the unsaponifiable fraction of cod-liver oil are always more or less yellow coloured, a fact which led them to consider the possibility of carotenoid pigments being involved. Lycopin and carotene were thus tested by these investigators and found to be inactive as a source of vitamin A, whilst a feeding experiment with xanthophyll was not undertaken as their material was not pure. It therefore seemed of interest to prepare a pure specimen of crystalline xanthophyll and to study its effect when fed in known amount to rats on a diet deficient in vitamin A compared with that of a potent source of this vitamin such as cod-liver oil.

## EXPERIMENTAL.

The crystalline xanthophyll was prepared from young nettle leaves by the method of Jørgensen and Stiles [1917] with certain modifications. Air-dried powdered nettle leaves were sieved so as to remove stalks and midribs and placed in the hot room at 40° for 3 days. The powder was then quickly re-ground, transferred to a large desiccator and dried over sulphuric acid *in vacuo* for a further 4 days. In the preliminary extraction with acetone 2 litres of solvent were used for each 500 g. of nettle powder as the filtrate still appeared considerably coloured after the 1½ litres recommended by Jørgensen and Stiles had been added. It was also found advantageous to recover the solvent held by the material after filtration under reduced pressure by expression in the hand press. By so doing more than a litre of extract was recovered from 1500 g. of the original material. Otherwise the method of Jørgensen and Stiles was closely followed. The crude xanthophyll was recrystallised three times from methyl alcohol, a little water being added to assist crystallisation and the flask placed in the refrigerator.

The yield was found to be little more than a third of that stated by these authors for the crude product. Allowing for the fact that our yield refers to the pure product, it is possible that there is a difference in xanthophyll content between the very young nettles we used as our material and the mature plant usually employed. The crystals were placed in an evacuated desiccator shaded from the light. They melted at 172° and gave the usual coloured solutions in organic solvents and answered the colour tests. Under the microscope the xanthophyll appeared as reddish yellow prisms.

*Feeding test.*

With the small quantity of crystalline xanthophyll available it was not possible to feed the substance to more than two albino rats. These animals were "run out" on the usual basal diet of this laboratory, vitamin D being supplied by direct irradiation for 10 minutes daily at a distance of 2 feet from the mercury vapour lamp. After their weights had become constant xanthophyll was administered at the rate of 3 mg. per head per day in the form of pellets. The pellets were made up with a small quantity of the basal mixture containing the correct dosage and were stored for the feeding period in a well-evacuated desiccator shaded from the light. These precautions are emphasised by the recent work of Schertz [1925], who finds that in the dry state at room temperature, xanthophyll is oxidised even more readily than carotene both in light and darkness. One pellet per day was fed by hand to each of the animals in advance of the basal ration, and very soon they were found to consume their dosage without objection. After a preliminary rise, both animals showed a marked decrease in weight which was not due to lack of appetite as the food intake was normal. Eventually both animals died and autopsies revealed the usual signs of vitamin A deficiency. Two control

animals fed 16 mg. of cod-liver oil in liquid paraffin gave a satisfactory growth response within 12 days, while both experimental animals died from vitamin A deficiency after 20 days' feeding with xanthophyll. It was therefore concluded that xanthophyll is ineffective in a daily dose of 3 mg. as a source of vitamin A.

#### SUMMARY.

Pure crystalline xanthophyll prepared from nettle leaves cannot be identical with vitamin A.

Our thanks are due to Sir F. G. Hopkins.

#### REFERENCES.

- Drummond (1919). *Biochem. J.* **13**, 81.  
Drummond and Coward (1920). *Biochem. J.* **14**, 668.  
Drummond, Channon and Coward (1925). *Biochem. J.* **19**, 1065.  
Jørgensen and Stiles (1917). *New Phytol.* Reprint No. 10.  
Palmer and Kempster (1919). *J. Biol. Chem.* **39**, 299, 313.  
Rosenheim and Drummond (1920). *Lancet*, i, 862.  
Schertz (1925). *J. Agric. Res.* **30**, 575.  
Stephenson (1920). *Biochem. J.* **14**, 715.

## XIV. THE TYROSINASE-TYROSINE REACTION.

### VI. PRODUCTION FROM TYROSINE OF 5:6-DIHYDROXY-INDOLE AND 5:6-DIHYDROXYINDOLE-2-CARBOXYLIC ACID—THE PRECURSORS OF MELANIN.

By HENRY STANLEY RAPER.

*From the Department of Physiology, University of Manchester.*

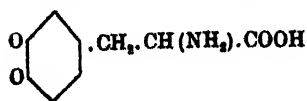
*(Received December 9th, 1926.)*

In the last communication of this series [Raper, 1926] it was shown that if a solution of the red pigment which is produced from tyrosine by tyrosinase is allowed to decolorise under suitable conditions, then 3:4-dihydroxyphenyl-alanine may be isolated from the solution. It was pointed out also that in the solution obtained by this method, the immediate precursor of melanin is present and that it possesses a structure in which the nitrogen is no longer in the amino form.

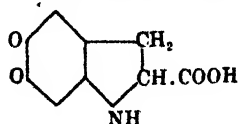
In the present paper an account is given of the identification of this precursor of melanin and the light it throws on the chemical changes involved in its production from tyrosine. It has not been possible to isolate the precursor itself because it is too easily oxidised, but its methyl derivative has been isolated in crystalline form and satisfactorily identified.

When the red solution obtained from tyrosine by the action of tyrosinase, as described previously [Raper, 1926], is allowed to decolorise *in vacuo* or in presence of sulphurous acid and then, after concentration in an atmosphere of  $\text{CO}_2$ , is methylated by means of methyl sulphate in an atmosphere of hydrogen, from the reaction product two crystalline methylated products can be obtained. One of them is a feeble base and the other an acid. The former is obtained principally when the red substance has been allowed to decolorise *in vacuo* and the latter when decolorisation takes place in the presence of sulphurous acid. Both products give colour reactions characteristic of indole derivatives.

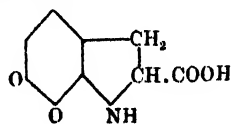
It was postulated in the paper already mentioned that the 3:4-quinone of phenylalanine (I) is formed prior to the production of the red substance in the tyrosinase-tyrosine reaction; and in attempting to determine the structure of the indole derivatives to which the red substance gives rise, it had to be considered whether in the formation of the indole ring the N of the amino group of the phenylalanine quinone attached itself in the 2 or 6 position in the benzene nucleus. Union in the 6 position would produce



I



II

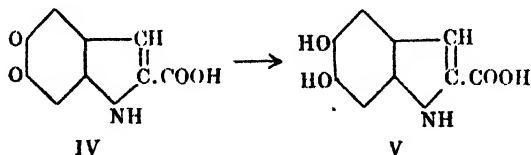


III



the 5 : 6-quinone of dihydroindole-2-carboxylic acid (II) and in the 2 position, the 6 : 7-quinone of dihydroindole-2-carboxylic acid (III). Further oxidation of II and III would result in the formation of the quinones of the corresponding indole carboxylic acids.

From the work of Jones and Robinson [1917] on the orientation of substituted catechol ethers it appeared to be most probable that the 5 : 6-quinone of indole-2-carboxylic acid would be formed, and it was suspected therefore that the methylated indole derivative with acid properties which was isolated from the products of oxidation was 5 : 6-dimethoxyindole-2-carboxylic acid, the quinone having undergone reduction to the corresponding dihydroxy-derivative in presence of sulphurous acid. It was also apparent that by loss of  $\text{CO}_2$  from this acid or from the quinone with subsequent reduction, 5 : 6-dihydroxyindole would be obtained and that the feeble base isolated after methylation was therefore likely to be 5 : 6-dimethoxyindole. Since 5 : 6-dimethoxyindole and its carboxylic acid had not been previously prepared they were therefore synthesised<sup>1</sup> and found to be identical in all respects with the two indole derivatives isolated from the oxidation products. This being established it is of interest to discuss the probable formula of the red substance, its mode of formation and the changes it undergoes when it becomes decolorised. The provisional formula for the red substance given previously [Raper, 1926] must now be considered to be very unlikely in view of the fact that in the presence of sulphurous acid it gives rise to 5 : 6-dihydroxyindole-2-carboxylic acid (V).



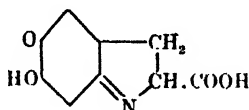
One way of explaining this transformation is to assume that the red substance is the 5 : 6-quinone of indole-2-carboxylic acid (IV) and that on reduction by sulphurous acid it yields the corresponding dihydroxy-compound. If this be the case then the decolorisation of the red substance which takes place in the absence of oxygen must be accompanied by a reduction as well as a loss of  $\text{CO}_2$  since the main product obtained is 5 : 6-dihydroxyindole.

This process is slower than the decolorisation brought about by sulphurous acid, and since unchanged tyrosine is present in the solution it could be explained as being brought about at the expense of the tyrosine, which would be oxidised.

Alternatively, the red substance may be considered to be the 5 : 6-quinone of dihydroindole-2-carboxylic acid (II) and to undergo slow autoreduction with the loss of  $\text{CO}_2$ , thus producing 5 : 6-dihydroxyindole. This however

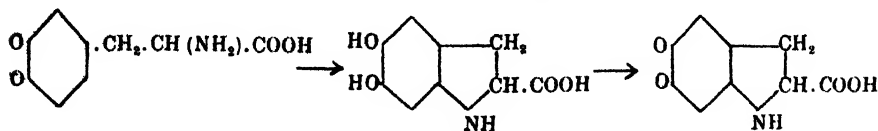
<sup>1</sup> The synthesis will be described elsewhere.

would render the decolorisation of the red substance in presence of sulphurous acid more difficult to understand, since on reduction it would yield 5 : 6-dihydroxydihydroindole-2-carboxylic acid, whereas the compound isolated after methylation is not a dihydroindole derivative. That a dihydroindole derivative is actually produced but subsequently oxidises in the air in the process of extraction subsequent to methylation is possible but unlikely. On the other hand the sulphurous acid may act simply as a catalyst and accelerate the autoreduction, which, proceeding more rapidly, would be less likely to result in the loss of  $\text{CO}_2$  and would thus give rise to the production of the dihydroxyindole carboxylic acid. When the autoreduction is not catalysed by  $\text{SO}_2$  and consequently proceeds relatively slowly there is more opportunity for the loss of  $\text{CO}_2$  and dihydroxyindole is produced. Another alternative formula for the red compound which gives it a *para*- instead of an *ortho*-quinonoid structure is the following:



This offers the same difficulty as II, namely, that it does not explain the production of an indole derivative rather than a dihydroindole derivative in presence of sulphurous acid. On the other hand, as in the case of (II) the difficulty disappears if it be assumed that the action of the sulphurous acid is catalytic and favours autoreduction. It might also be expected to have a yellow rather than a red colour which is more characteristic of the *ortho*-quinonoid grouping.

From these considerations it appears most probable that the red compound is the 5 : 6-quinone of dihydroindole-2-carboxylic acid and that this substance undergoes autoreduction and loss of  $\text{CO}_2$  slowly at the ordinary temperature and more rapidly on heating with production of 5 : 6-dihydroxyindole. In the presence of sulphurous acid, however, the autoreduction proceeds more rapidly and consequently the loss of  $\text{CO}_2$  is much less marked. This results in the production mainly, therefore, of 5 : 6-dihydroxyindole-2-carboxylic acid. The experimental results show that in presence of sulphurous acid this is almost the only product produced, only traces of the base being obtained. In the absence of sulphurous acid, however, the base forms almost the entire product which can be isolated, only traces of the acid being obtained. If these views be correct, then the formation of the red substance from the 3 : 4-quinone of phenylalanine may be represented as follows:



The first stage may be considered as analogous to the formation of an anilino-quinone, the second is brought about by the oxidation of the dihydroxydihydroindole carboxylic acid by the enzyme. This is probably rapid since it is analogous to the oxidation of 3:4-dihydroxyphenylalanine to the corresponding quinone which occurs in an earlier stage of the tyrosinase reaction and is known to take place rapidly.

The presence of dihydroxyphenylalanine in small amount along with the indole derivatives is accounted for partly by its being an intermediate between tyrosine and the quinone of phenylalanine, and partly by the reduction of the latter by sulphurous acid when this is used to accelerate the decolorisation of the red substance. It has already been suggested by the author that 3:4-dihydroxyphenylalanine is the first product produced from tyrosine when it is oxidised by tyrosinase and its isolation and identification as a product of the reaction, together with other evidence put forward in the last communication of this series, rendered this very probable. But it seemed to be important, in order to place the matter beyond doubt, to demonstrate that the red substance produced when tyrosinase acts on 3:4-dihydroxyphenylalanine will undergo transformation to yield the same indole derivative that tyrosine produces under the same conditions. This has now been done. When the red substance, obtained from 3:4-dihydroxyphenylalanine by the action of the enzyme, was allowed to decolorise *in vacuo*, it was possible to isolate 5:6-dimethoxyindole from the resulting products by the same procedure that was used in the case of tyrosine.

These results place on a more secure basis the hypothesis first advanced by Happold and Raper [1925] that tyrosinase produces *ortho*-quinones from those phenols which bring about deamination when acted upon by the enzyme in presence of an amino-acid. This view has been extended to oxidases in general which will act upon substances containing the catechol grouping by Szent-Györgyi [1925], who has also made it the basis of a theory as to the nature of oxidases. Experiments in progress in the author's laboratory indicate very clearly that tyrosinase acting on phenol and *p*-cresol produces *o*-quinones, for it has been found that when the enzyme is allowed to act on either of these phenols in the presence of aniline, considerable amounts of the anilino-quinones are obtained. The complete results of these experiments will be published shortly.

It is presumed from the results described in this paper that melanin production occurs by the oxidation in the air of 5:6-dihydroxyindole and to a lesser extent of 5:6-dihydroxyindole-2-carboxylic acid. These substances have not been synthesised, but there is no doubt that they would be extremely easily oxidised in the air and especially in slightly alkaline solution—the optimum condition for melanin production from tyrosine. Attempts to remove the methyl groups from dimethoxyindole or its carboxylic acid have been fruitless as might be expected, since this can only be done in acid solution, under which conditions both the indole and its carboxylic acid yield tarry

products [*vide* Perkin and Rubenstein, 1926]. Under slightly alkaline conditions the oxidation of the traces of dihydroxyphenylalanine which are found amongst the products of action of the enzyme would contribute to melanin formation also [Bloch and Schaaf, 1925].

The formation of melanin from dihydroxyindole and its carboxylic acid is of considerable interest from the point of view of the normal production of melanin in pigment cells and in melanotic tumours. From time to time observations have been made which indicated that melanin on destructive decomposition yielded cyclic compounds containing nitrogen which give the pine-shaving reaction. This has been taken by some to mean that melanins were possibly formed from blood pigment. Evidence against this view has been collected by von Fürth [1912] and it is now not generally accepted. On the other hand it has led to the view (von Fürth) that tryptophan might be the mother-substance of melanin in pigment-producing cells. The isolation from the urine in a case of melanotic sarcoma of a substance which gave indole reactions has also given support to this view [Eppinger, 1910] and especially so since administration of tryptophan increased the amount of this substance excreted, whereas tyrosine did not. On the other hand, Bloch and Schaaf [1925] have advanced the view that melanin is formed from 3:4-dihydroxyphenylalanine by a specific enzyme "dopa oxydase," and a structure for the pigment is given in which its formation is based on the production of the 3:4-quinone of phenylalanine, two molecules of which are represented as condensing with one molecule of the 3:4-quinone of phenylacetaldehyde.

It would appear from the observations recorded in this paper that the cyclic group containing nitrogen is already present in the precursors of melanin and that it is likely to persist when melanin has been formed by oxidation of these precursors in the air or by means of tyrosinase. Whether, however, the melanin obtained from tyrosine by oxidation with tyrosinase is identical with that found in pigment cells cannot be yet considered as proven. It is on the whole unlikely, since Abderhalden and Guggenheim [1907, 1908] have shown that peptides containing tyrosine will also produce melanin when oxidised by the enzyme and it is likely that the peptide linkage will persist in the final product. It does however seem probable that the reactions leading to the formation of the indole nucleus from tyrosine, or peptides containing it, as described in this paper, are an essential preliminary in the natural production of melanin.

#### EXPERIMENTAL.

The enzyme used in the following experiments was prepared from the mealworm (*Tenebrio molitor*) as described previously by the author [Raper, 1926].

##### I. *Production of 5:6-dimethoxyindole from tyrosine.*

1 g. of tyrosine is dissolved in 500 cc. hot water and the solution diluted to 2 l. in a large stoppered bottle.

The temperature is adjusted to 26–30° by cooling or warming as required and 100 cc. of the enzyme preparation are added. The  $p_H$  is now adjusted to between 6 and 6.5 by the cautious addition of 1 % acetic acid. Oxygen is bubbled through the solution for 1–2 minutes and the bottle is stoppered and well shaken. About every half-hour oxygen is bubbled through the liquid and it is frequently shaken. The solution quickly becomes red and in 2 to 4 hours most of the enzyme has been precipitated. To ensure complete precipitation 10 cc. of 1 % acetic acid are added and after standing for a few minutes the solution is filtered quickly, using several large funnels. The deep red filtrate is placed in a large filter flask and the flask evacuated. The liquid is well shaken to remove as much dissolved oxygen as possible and hydrogen is then admitted. It is now again evacuated and re-filled with hydrogen. The red liquid is allowed to stand at the ordinary temperature under hydrogen until the red tint has been lost and replaced by a pale brown. 10 cc. of a saturated solution of  $SO_2$  are now added to minimise oxidation in the succeeding operations. The solution is now filtered to remove a small amount of brown deposit and placed in a flask from which it can be displaced gradually and continuously to a large distillation flask while at the same time being kept under hydrogen. It is then concentrated by vacuum distillation in a stream of  $CO_2$  until it measures about 20 cc. The reaction products in this solution together with unchanged tyrosine are now methylated in an atmosphere of hydrogen. The flask is detached and fitted with a reflux condenser, inlet and outlet tubes for hydrogen and a dropping funnel. The flask is evacuated and filled with hydrogen, this process being repeated once to ensure complete absence of oxygen.

With a slow stream of hydrogen passing through the flask, the methylation is carried out by first adding 10 cc. of 20 % NaOH, shaking gently till the contents of the flask are all in solution, and then adding 3.5 cc. dimethyl sulphate in two portions (2.5 and 1 cc.). After each addition the flask is vigorously shaken. It is now heated on the water-bath for an hour, cooled and the brown alkaline solution extracted three times with ether. The ether extract is washed with a small amount of water. On removal of the ether by distillation, from 100 to 150 mg. of the crystalline dimethoxyindole have usually been obtained. It was recrystallised from a small volume of alcohol and was pure after two crystallisations, m.p. 154–5°. Mixed with synthetic 5:6-dimethoxyindole the m.p. remained unchanged.

*Analysis.* Found: C, 67.6 %; H, 6.2 %; N, 8.3 %.

Calculated: C, 67.8 %; H, 6.2 %; N, 7.9 %.

If the alkaline solution resulting from the methylation after extraction with ether is acidified to Congo red and again shaken out several times with ether, traces of an acid are obtained which on crystallisation from benzene containing a small amount of acetone melts at 202–3° with evolution of gas. This acid is therefore 5:6-dimethoxyindole-2-carboxylic acid (see next section).

A trace of the base dissolved in alcohol and mixed with a few drops of *p*-dimethylaminobenzaldehyde in alcohol gave a violet colour on adding a drop of 20 % HCl. Addition of a trace of sodium nitrite changed the colour to a deep crimson. The substance gave a reddish violet colour with sodium nitroprusside and caustic soda which was changed to a pure blue on adding acetic acid. Mixed with glyoxylic acid and run on to concentrated sulphuric acid the substance gave a red colour at the junction of the two liquids.

## II. Production of 5 : 6-dimethoxyindole-2-carboxylic acid from tyrosine.

The procedure to be carried out up to the stage at which the solution of the red pigment is obtained is the same as described in the previous section. Instead of keeping the red solution under hydrogen, it is mixed with 50–60 cc. of a saturated solution of SO<sub>2</sub> and allowed to stand for 1 to 2 days in a stoppered bottle. The pale yellow solution is now concentrated *in vacuo* in a large distilling flask and when reduced to about 20 cc. is methylated in an atmosphere of hydrogen exactly as described above. The alkaline solution obtained after methylation is extracted with ether and usually yields a trace of crystalline substance which gives indole reactions and is presumably 5 : 6-dimethoxyindole. On acidifying and shaking out several times with ether a substance is obtained which is left as a vitreous residue after evaporation of the ether. It crystallises on allowing it to stand with water for a few hours and is crude 5 : 6-dimethoxyindole-2-carboxylic acid. The yield has usually been about 0.2 g. from each gram of tyrosine initially taken. It was purified by dissolving in the minimal volume of acetone and adding 15 volumes of hot benzene, filtering at once and allowing to stand. It crystallised in flattened, rhombic prisms, m.p. 202–3° with evolution of gas. Mixed with synthetic 5 : 6-dimethoxyindole-2-carboxylic acid the m.p. remained unchanged.

*Analysis.* Found: C, 59.8 %; H, 5.1 %; N, 6.6 %.

Calculated: C, 59.7 %; H, 5.0 %; N, 6.3 %.

In the *p*-dimethylaminobenzaldehyde reaction it gives a less intense violet colour than 5 : 6-dimethoxyindole. If it be heated above its m.p. until CO<sub>2</sub> evolution ceases and the residue then tested, the indole reaction is more marked and similar in all respects to that given by pure 5 : 6-dimethoxyindole. The acid or base dissolved in glacial acetic acid gives a yellow colour when one drop of nitric acid is added (brucine test).

## III. Production of 5 : 6-dimethoxyindole from 3 : 4-dihydroxyphenylalanine.

1 g. dihydroxyphenylalanine prepared from *Vicia faba* according to the directions of Guggenheim [1913] was dissolved in 2 l. water, cooled to 27° and 70 cc. of the enzyme solution added. The *p<sub>H</sub>* was adjusted to 6.5 and the solution saturated with oxygen. The solution rapidly became a deep red and in 10 minutes the enzyme had precipitated out. After half-an-hour 10 cc. of 1 % acetic acid were added and the solution was filtered. The red filtrate was

dealt with exactly as described in the first section. After methylation 136 mg. of the crude base were obtained. This was recrystallised twice from alcohol and had m.p. 154–5°. Mixed with synthetic 5:6-dimethoxyindole the m.p. remained the same. It gave the typical indole reaction with *p*-dimethylamino-benzaldehyde.

#### SUMMARY.

1. 5:6-Dihydroxyindole and 5:6-dihydroxyindole-2-carboxylic acid have been identified as products of the action of tyrosinase on tyrosine and are to be regarded as the immediate precursors of melanin.

2. 5:6-Dihydroxyindole is the chief product produced when the red pigment which is characteristic of the tyrosinase reaction undergoes intra-molecular change. 5:6-Dihydroxyindole carboxylic acid is however produced principally when the red substance is allowed to decolorise in the presence of sulphurous acid.

3. 3:4-Dihydroxyphenylalanine also gives rise to 5:6-dihydroxyindole under the same conditions that result in its production from tyrosine. This confirms the previous suggestion that 3:4-dihydroxyphenylalanine is the first intermediate product in the tyrosinase-tyrosine reaction.

4. The formula previously given to the red pigment produced from tyrosine has been revised in view of the further evidence presented in this paper. It is probably the 5:6-quinone of dihydroindole-2-carboxylic acid.

5. The production of melanin is discussed.

I wish to thank Mr T. Kirkman for the preparation of the dihydroxy-phenylalanine used in this work, and the Government Grant Committee of the Royal Society for a grant in aid of expenses. I wish also to express my appreciation of the kindness of my colleague, Prof. R. Robinson, for his assistance on several points in connection with the theoretical aspects of this work.

#### REFERENCES.

- Abderhalden and Guggenheim (1907). *Z. physiol. Chem.* **54**, 331.  
— — (1908). *Z. physiol. Chem.* **57**, 329.  
Bloch and Schaaf (1925). *Biochem. Z.* **162**, 181.  
Eppinger (1910). *Biochem. Z.* **28**, 181.  
Von Fürth (1912). *Probleme d. pathol. u. physiol. Chem.* (Leipzig).  
Guggenheim (1913). *Z. physiol. Chem.* **88**, 276.  
Happold and Raper (1925). *Biochem. J.* **19**, 92.  
Jones and Robinson (1917). *J. Chem. Soc.* **111**, 903.  
Perkin and Rubenstein (1926). *J. Chem. Soc.* **130**, 357.  
Raper (1926). *Biochem. J.* **20**, 735.  
Szent-Györgyi (1925). *Biochem. Z.* **162**, 399.

# XV. THE CONSTITUTION AND SYNTHESIS OF SPERMIDINE, A NEWLY DISCOVERED BASE ISOLATED FROM ANIMAL TISSUES.

BY HAROLD WARD DUDLEY, OTTO ROSENHEIM  
AND WALTER WILLIAM STARLING.

*From the National Institute for Medical Research, Mount Vernon,  
Hampstead, N.W. 3.*

*(Received December 10th, 1926.)*

THE presence of an unidentified base in the mother-liquors after the isolation of spermine phosphate from tissue extracts was originally observed by M. C. Rosenheim and O. Rosenheim during their early work on spermine and has already been reported [Dudley, Rosenheim and Rosenheim, 1924].

This base has been given the name *spermidine* since it not only occurs in association with spermine but also has been found to be structurally related to the latter substance. Its phosphate is much more soluble than that of spermine and crystallises from the 25 % alcoholic mother-liquor, after the removal of spermine phosphate, on increasing the concentration of alcohol to about 50 %.

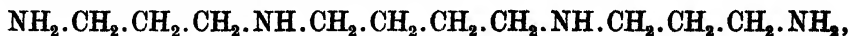
The material employed in the present investigation was isolated by this procedure during the large scale preparation from ox pancreas of the spermine used in our recently published work on the constitution of the latter base [Dudley and Rosenheim, 1925; Dudley, Rosenheim and Starling, 1926]. The yield of spermidine phosphate is extremely small, being only one-tenth of that of spermine phosphate, namely about 2 g. from 100 kg. pancreas. Only 10 g. of spermidine phosphate were available but, aided by our recently acquired knowledge of the constitution of spermine, we were able to arrive at a probable constitutional formula for the base, and then to prove its correctness by synthesis.

The properties of spermidine are very similar to those of spermine. This is demonstrated in a general way by the fact that it accompanies spermine through all the stages of isolation from tissue extracts by the three methods already described [Dudley, Rosenheim and Rosenheim, 1924]. Like spermine it gives a pyrrole reaction, it appears in the lysine fraction on applying the method of Kossel and Kutscher, its phosphotungstate is insoluble in acetone, it is optically inactive, it does not reduce permanganate in faintly acid solution

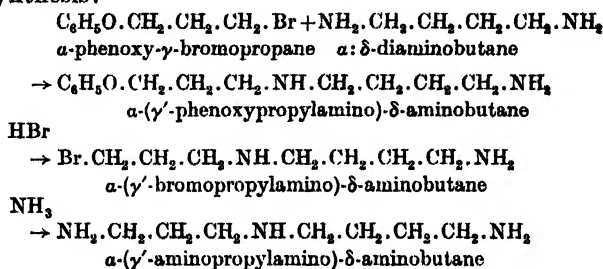


and it yields the characteristic semen-like odour when a solution of its chloroaurate is treated with magnesium.

Analyses of its salts gave figures very close to those of the corresponding salts of spermine but indicated that the ratio of carbon to nitrogen in spermidine is lower than in spermine. The molecular weight of the *m*-nitrobenzoyl derivative of the base, considered in conjunction with the analyses, established the molecular formula of spermidine as  $C_7H_{19}N_3$ . In view of the striking similarity between spermidine and spermine it was considered probable, therefore, that spermidine might be  $\alpha$ -( $\gamma'$ -aminopropylamino)- $\delta$ -aminobutane,  $NH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH_2$ , a formula which is derived from that of spermine, whose structure we have shown to be



by the replacement of one of the terminal  $\gamma$ -aminopropylamino-groups by an atom of hydrogen. The correctness of this conjecture was proved by the following synthesis:



The final compound proved to be in all respects identical with natural spermidine, whose constitution is thus established.

#### EXPERIMENTAL.

##### *Spermidine phosphate.*

An equal volume of alcohol is added to the concentrated mother-liquor from the preparation of spermine phosphate [Dudley, Rosenheim and Rosenheim, 1924].

Spermidine phosphate crystallises from this solution in lustrous plates, usually contaminated with a small amount of spermine phosphate. The easily soluble spermidine phosphate is dissolved in the minimum requisite quantity of cold water and the spermine phosphate, which remains suspended in the solution, is filtered off. To the filtrate, warmed on the water-bath, alcohol is added until a faint permanent turbidity is produced. On allowing the solution to cool spermidine phosphate crystallises. It can be recrystallised from a hot 20 % solution in water. The crystals so obtained are similar in appearance to those of cholesterol and entirely different from the characteristic spermine phosphate.

Heated in a melting-point tube spermidine phosphate begins to shrink at  $150^\circ$  and melts at  $207-9^\circ$  to an opaque fluid which begins to froth at  $218-20^\circ$  without discolouring.

*Analysis.* 0.2944 g. lost 0.0463 g.  $H_2O$  at  $108^\circ$

Found:	$H_2O$ , 15.73 %
Calc. for $(C_7H_{15}N_3)_2 \cdot 3H_2PO_4 \cdot 6H_2O$ :	$H_2O$ , 15.61 %
0.2341 g. (dry) gave 0.1336 g. $Mg_2P_2O_7$	
0.16155 g. (dry) gave $NH_3 = 16.72$ cc. $N/10$ $H_2SO_4$ (Kjeldahl)	
Found:	P, 15.89 %; N, 14.49 %
Calc. for $(C_7H_{15}N_3)_2 \cdot 3H_2PO_4$ :	P, 15.92 %; N, 14.38 %

### *Spermidine picrate.*

This salt was prepared by adding excess of sodium picrate solution to a solution of spermidine phosphate. The picrate, which is sparingly soluble in water, when recrystallised from this solvent is obtained as lemon-yellow needles. Under the microscope these are seen to be composed of long thin laminated plates. Under certain conditions of concentration and rate of cooling the crystals formed are broader, being composed of the same thin laminae fused together and appearing to the naked eye as thin plates. Spermidine picrate melts at  $210-12^\circ$  to an oily liquid which almost immediately decomposes and froths slowly up the tube.

*Analysis.* 0.0473 g. (dried at  $102^\circ$ ) gave 0.0924 g. nitron picrate

Found:	picric acid, 82.68 %
Calc. for $C_7H_{15}N_3 \cdot 3C_6H_3O_7N_3$ :	picric acid, 82.58 %

### *Spermidine hydrochloride.*

3.55 g. spermidine picrate were treated on the water-bath with a mixture of 45 cc. absolute alcohol, 5 cc. concentrated hydrochloric acid and 2.5 cc. water. The picrate dissolved in the hot solution and the hydrochloride crystallised in needles on cooling. The crystals were redissolved by heating and 25 cc. acetone were added. The hydrochloride crystallised from the cooled solution and was filtered off, washed with acetone and dried in a vacuum desiccator: yield 1.04 g. On recrystallisation from a mixture of 50 cc. absolute alcohol and 8.5 cc. concentrated hydrochloric acid it was obtained in glistening thin plates, containing no water of crystallisation.

Spermidine hydrochloride is only very slightly hygroscopic; it is quite stable in a relatively dry atmosphere but slowly deliquesces when the humidity is high.

*Analysis.* Dried at  $100^\circ$ . 0.1396 g. gave 0.1717 g.  $CO_2$  and 0.1055 g.  $H_2O$

" 0.1331 g. gave  $NH_3 = 15.75$  cc.  $N/10$   $H_2SO_4$  (Kjeldahl)

" 0.1258 g. gave 0.2121 g.  $AgCl$

Found: C, 33.54 %; H, 8.46 %; N, 16.53 %; Cl, 41.71 %

Calc. for  $C_7H_{15}N_3 \cdot 3HCl$ : C, 33.01 %; H, 8.64 %; N, 16.50 %; Cl, 41.85 %

\* Slightly high owing to incomplete reduction of oxides of nitrogen during the combustion.

### *Spermidine chloraurate.*

0.1 g. spermidine hydrochloride was dissolved in 7 cc. water. On addition of 5 cc. 10 % "gold chloride" solution an immediate crystalline precipitate appeared. After standing at  $2^\circ$  for 2 hours the chloraurate was filtered off and recrystallised from 6 cc. 1 % hydrochloric acid.

It forms thin, lustrous, golden yellow plates; m.p. 220–2° with decomposition.

When a more dilute solution is allowed to stand for some time at 2° the salt crystallises in long thin needles with the same melting-point as the plates.

*Analysis.* 0.1833 g. dried at 100°, gave 0.0930 g. Au.

Found:	Au, 50.73 %
Calc. for $C_7H_{16}N_3 \cdot 3HAuCl_4$ :	Au, 50.72 %

#### *m*-Nitrobenzoylspermidine.

To 0.15 g. spermidine hydrochloride, dissolved in 6 cc. *N* NaOH, 0.9 cc. molten *m*-nitrobenzoyl chloride was added and the mixture was vigorously stirred in a small beaker. As soon as the reaction of the liquid became nearly neutral 2 cc. *N* NaOH were added and this addition was repeated as necessary. A sticky white mass was formed which slowly became more solid. When the substance became plastic it was pressed out to a thin cake, the mother-liquor was poured off and 2 cc. *N* NaOH were added. After standing overnight the crude *m*-nitrobenzoyl derivative had become friable and was broken up to a powder, filtered off and washed with water: yield (air-dried) 0.336 g.

It was suspended in 20 cc. absolute alcohol. When warmed on the water-bath the solid melted to an oil which then slowly dissolved. To the filtered solution 20 cc. water were added, the turbid solution was warmed until clear and set aside for a day. The *m*-nitrobenzoyl derivative had then crystallised in aggregates of thick needles. These were filtered off and washed with 50 % alcohol: yield (air-dried) 0.298 g. The substance melted at 102° to a turbid liquid which slowly frothed up the tube without charring at higher temperatures. On recrystallising under the same conditions it behaved in the same manner and the melting-point was unchanged. It was then dried in a vacuum desiccator over  $H_2SO_4$ . On warming the substance (0.28 g.) with 25 cc. absolute alcohol the oil which formed at first rapidly changed to a white solid which went very slowly into solution. From this solution, after standing overnight, the substance crystallised in balls of radiating needles. These were collected and again recrystallised from 20 cc. absolute alcohol. On heating the substance with alcohol it no longer formed an oil but remained solid and dissolved very slowly. 0.2155 g. was obtained which melted at 148–50°. In all probability the low-melting product originally obtained was a hydrate.

#### *Molecular weight determination* (Rast).

0.0042 g. was dissolved in 0.0378 g. camphor.  $\Delta$  7.5°, m.w. 592.

Calc. for  $C_7H_{16}N_3$  ( $CO \cdot C_6H_4 \cdot NO_2$ )<sub>3</sub>: m.w. 592.

*Analysis.* 0.1590 g. (dried for 2 days *in vacuo* over  $H_2SO_4$ ) gave 20.8 cc.  $N_2$  at 20° and 728 mm. (Dumas).

Found:	N, 14.37 %
Calc. for $C_7H_{16}N_3$ ( $CO \cdot C_6H_4 \cdot NO_2$ ) <sub>3</sub> :	N, 14.20 %

Spermidine forms a chloroplatinate and a mercurichloride which are very soluble. It also yields a crystalline phenylisocyanate derivative, but these compounds are not suitable for purposes of identification.

## SYNTHESIS OF SPERMIDINE.

 *$\alpha$ -( $\gamma'$ -Phenoxypropylamino)- $\delta$ -aminobutane hydrobromide.*

This substance was isolated as a by-product from the preparation of  $\alpha$ : $\delta$ -bis( $\gamma'$ -phenoxypropylamino)-butane hydrobromide used in the synthesis of spermine [Dudley, Rosenheim and Starling, 1926]. The mother-liquor from the recrystallisation of the latter substance was concentrated and, after adding alcohol, was poured into 10 volumes of ether. The white crystalline precipitate (5.8 g.) was recrystallised from 96 % alcohol, separating in thin rectangular plates, M.P. 271–3°.

*Analysis.* 0.1950 g. gave 0.1896 g. AgBr.

0.3648 g. gave  $\text{NH}_3 = 19.6$  cc.  $N/10$   $\text{H}_2\text{SO}_4$  (Kjeldahl)

Found: Br, 41.38 %; N, 7.52 %

Calc. for  $\text{C}_{13}\text{H}_{24}\text{ON}_2\text{Br}_2$ : Br, 41.67 %; N, 7.30 %

 *$\alpha$ -( $\gamma'$ -Bromopropylamino)- $\delta$ -aminobutane hydrobromide.*

4.6 g. of the above phenoxy-compound were heated in a sealed tube at 100° for 12 hours with 25 cc. HBr solution (Sp. G. 1.7). On shaking the clear solution with ether to remove phenol a crystalline substance was precipitated; this was filtered off and washed with alcohol and ether. After three recrystallisations from absolute alcohol 2.4 g. of prismatic needles were obtained: M.P. 234–5°, which proved to be the required substance.

*Analysis.* 0.0604 g. gave 0.1014 g. AgBr.

Found: Br, 64.97 %

Calc. for  $\text{C}_7\text{H}_{19}\text{N}_2\text{Br}_2$ : Br, 64.69 %

 *$\alpha$ -( $\gamma'$ -Aminopropylamino)- $\delta$ -aminobutane picrate (synthetic spermidine picrate).*

1.5 g. of the above bromo-compound were heated in a sealed tube at 100° for 4½ hours with 19.4 g. of an alcoholic solution of ammonia (16 % by weight). The reaction mixture was then evaporated *in vacuo*. The white solid remaining was dissolved in water, potassium hydroxide was added to give a concentration of about 50 % and the solution was then steam-distilled until the distillate gave only a faint cloud on adding picric acid solution. The first fraction of the distillate (200 cc.) was boiled for 5 minutes to expel ammonia. The whole distillate was then neutralised with hydrochloric acid and evaporated to small bulk. Addition of sodium picrate solution precipitated a picrate which, after recrystallisation, was found to be identical with the picrate of natural spermidine. From this picrate the *hydrochloride*, *chloroaurate* and *m-nitrobenzoyl derivative* were prepared. These compounds displayed exactly the same properties as those of natural spermidine. Mixed samples of the corresponding derivatives of the synthetic and natural bases showed no depression of melting points.

*Analyses of synthetic spermidine salts.*

0.0448 g. picrate gave 0.0872 g. nitron picrate

Found: picric acid, 82.38 %

Calc. for  $\text{C}_7\text{H}_{19}\text{N}_2 \cdot 3\text{C}_6\text{H}_3\text{O}_7\text{N}_3$ : picric acid, 82.58 %

0.1558 g. chloroaurate gave 0.0784 g. Au.

Found: Au, 50.32 %

Calc. for  $\text{C}_7\text{H}_{19}\text{N}_2 \cdot 3\text{HAuCl}_4$ : Au, 50.72 %

As the problem of identifying minute amounts of spermidine may arise in the future a few remarks in this connection may not be out of place. In extracts of animal tissues the base will usually be found in association with spermine. The only feasible way of separating these two substances is to convert them into phosphates and then to remove the very insoluble spermine salt. It is essential to separate the two bases completely in this way before preparing the picrates, since it appears to be impossible to separate the two picrates by recrystallisation. There is a characteristic difference between the melting-points of the picrates: that of spermidine forms an oil at  $210-12^{\circ}$  which decomposes almost immediately, whilst that of spermine gradually darkens until it becomes black, softening and decomposing at  $248-50^{\circ}$  without first melting. The *m*-nitrobenzoyl derivative of spermidine is valuable for identification of the base since a molecular weight determination by Rast's method [1923] requires no more material than is used in an ordinary melting-point determination and since there is a considerable difference between the molecular weights of the corresponding derivatives of spermine and spermidine. The preparation and analysis of a chloroaurate is not decisive on account of the very slight differences in melting-point and gold content of these salts of the two bases.

It was pointed out in our communication on the structure of spermine [Dudley, Rosenheim and Starling, 1926] that this base is the first example of a naturally occurring compound of the type  $\text{NH}_2\cdot\text{R}\cdot\text{NH}\cdot\text{R}'\cdot\text{NH}\cdot\text{R}\cdot\text{NH}_2$  and at the same time the first derivative of trimethylenediamine definitely identified among natural products. It is interesting to find the trimethylenediamino-group in a second naturally occurring base, spermidine, which is the first example found in nature of the closely related type



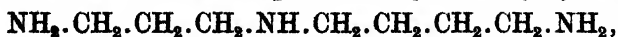
It seems reasonable to suspect that trimethylenediamine itself may be detected among the bases extracted from fresh tissues; in this connection the fact may be recalled that tetramethylenediamine has already been definitely identified in the mother-liquors after the isolation of spermine from yeast [Dudley, Rosenheim and Rosenheim, 1924].

It is hardly necessary to state that the chemical procedures incidental to the isolation of spermine and spermidine cannot have produced artificially the latter from the former. The two bases undoubtedly exist together in animal tissues, and their close chemical relationship strongly suggests that they may be metabolically related.

#### SUMMARY.

1. A hitherto undescribed base, having the molecular formula  $\text{C}_7\text{H}_{19}\text{N}_3$ , has been isolated from extracts of animal tissues.
2. In occurrence and constitution it is allied to spermine and has therefore been given the name *spermidine*.

3. The structural formula of spermidine, proved by synthesis, is



*i.e.*  $\alpha$ -( $\gamma'$ -aminopropylamino)- $\delta$ -aminobutane.

## REFERENCES.

- Dudley and Rosenheim (1925). *Biochem. J.* **19**, 1032.  
Dudley, Rosenheim and Rosenheim (1924). *Biochem. J.* **18**, 1263.  
Dudley, Rosenheim and Starling (1926). *Biochem. J.* **20**, 1082.  
Rast (1923). *Z. physiol. Chem.* **126**, 111.

## XVI. STUDIES ON THE GROWTH OF YEAST.

### III. A FURTHER STUDY ON THE INFLUENCE OF VOLUME OF MEDIUM EMPLOYED.

By GEOFFREY LEWIS PESKETT.

*From the Biochemical Laboratory, Oxford.*

*(Report to the Medical Research Council.)*

*(Received December 18th, 1926.)*

IN a previous communication [1925, 1] I have recorded the growth of single-cell cultures of yeast in various volumes of medium. At the time of publication of those experiments I calculated that in the series of volumes employed I had included the different volumes of medium per yeast cell inoculated which were used by Liebig and Pasteur. I subsequently found that owing to an error in all my calculations the volumes which I had actually studied were not in cubic millimetres as recorded, but in thousandths of a cubic millimetre. I had therefore only studied concentrations of yeast equal to and greater than those used by Pasteur, and even in the largest volumes I had not employed such a low concentration of yeast as did Liebig. On this account the experimental evidence of the communication did not justify any conclusions as to the causes of the differences in growth observed by Pasteur and Liebig. The more general conclusions concerning the relationship between volume and growth are obviously not affected by any alterations in the units in which the volumes were recorded. In the experiments detailed below I have extended the range of volumes to include that which corresponds with the concentration of yeast employed by Liebig.

#### EXPERIMENTAL.

The experimental details will be found in the previous paper [Peskett, 1925, 1]. The yeast used was the same strain of *Saccharomyces cerevisiae*, and the cells whose growth was studied in these experiments were washed before inoculation by the technique described elsewhere [Peskett, 1925, 2]. The stock and parent cultures were grown on medium 5. All cultures were grown at 25°. Medium 1 consisted of the basal salt mixture plus 50 g. per litre commercial cane sugar; in medium 2 the latter was replaced by recrystallised cane sugar, and medium 5 was composed of medium 2 plus 10.0 cc. per litre yeast extract.

*Volumes.* In Exp. 1 the volumes were approximately 5.0 and 0.5 mm.<sup>3</sup>. If a yeast cell can be considered as a sphere of 10  $\mu$  diameter, *i.e.* about

$5 \times 10^{-7}$  mm.<sup>3</sup> volume, then single cells in these volumes would correspond to concentrations of 0.01 mm.<sup>3</sup> and 0.1 mm.<sup>3</sup> of yeast per 100 cc. of medium respectively. These concentrations are approximately those used by Liebig and Pasteur, as will be seen from the discussion in my previous communication [1925, 1, p. 471].

In Exp. 2 the volumes were 39.7 mm.<sup>3</sup> and 0.04 to 0.01 mm.<sup>3</sup>, *i.e.* roughly 10 times larger than Liebig's, and 10 times smaller than Pasteur's per yeast cell; in addition three cultures were studied in which the volumes were intermediate.

*Preparation of the cultures.* For use in Exp. 1 a silica capillary pipette was calibrated to deliver 5.0 mm.<sup>3</sup> of mercury determined by weighing. A suspension in medium 1 of washed yeast cells was prepared of such a dilution that 5.0 mm.<sup>3</sup> of it could be distributed in 10 drops of approximately equal diameter, a few of which would contain a single cell. Thus hanging-drops were prepared of about 0.5 mm.<sup>3</sup> volume, some of which contained a single cell.

The 5.0 mm.<sup>3</sup> drops of medium 1 were measured from the capillary pipette on to slides, each of which had a deep circular depression about 13/16 inch in diameter. The drops were each inoculated with a single washed cell transferred by means of a capillary pipette from hanging-droplet preparations in medium 2 which had been prepared previously. Particular care was taken to search these droplets thoroughly, and in every case only those were used which contained one cell beyond all doubt. Immediately after inoculation the cultures were covered by a cover-slip sealed in position with paraffin wax. The under surface of the cover-slips had been treated before sterilisation with a commercial preparation to prevent fogging owing to condensation of water. This did not come in contact with the cultures and therefore did not vitiate the results.

The error in measuring the drops in Exp. 1 was fairly considerable. The pipette delivered 4.9 mm.<sup>3</sup> of mercury  $\pm$  0.07 mm.<sup>3</sup>. In measuring medium this error must at least have doubled owing to wetting of the surface by the fluid. Thus the probable error was at least 3 % for the larger drops, and it may have increased up to 10 % for the smaller though every effort was made to keep the diameters of the latter constant within 0.1 mm.

In Exp. 2 the largest volumes were measured (1 drop) from a Dreyer pipette which delivered 252 drops of medium 1 per 10.0 cc. The volumes of the other drops were calculated from measurements of their diameter and depth. The largest and intermediate drops were set up and inoculated as in Exp. 1, the smallest were prepared in the usual manner from a suspension of washed cells in medium 1.

Every precaution was taken to prevent contamination of the cultures.

*Observation and bacterial control.* The cultures were examined at intervals of about 4 hours, and the growth recorded. In the case of the larger drops it was often necessary to warm the cover-slip to dispel the thin film of water which had condensed thereon, in order to get clear definition of the cells under



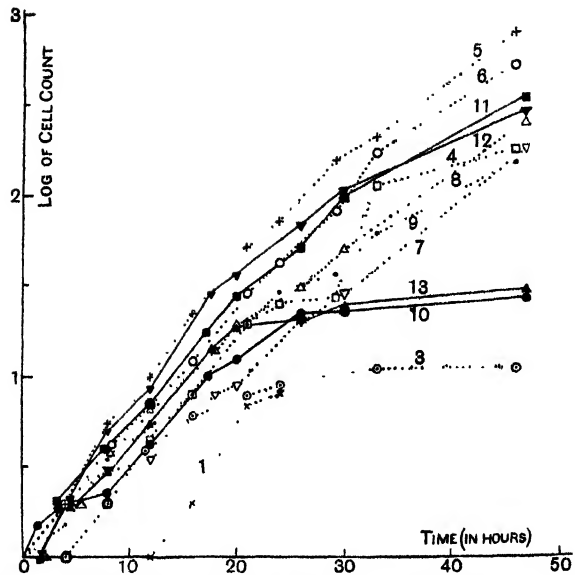


Fig. 1. Growth of washed single cells in medium composed of recrystallised salts and commercial cane sugar.

Volume (mm.<sup>3</sup>)  
5.0 —————  
0.5 . . . . .

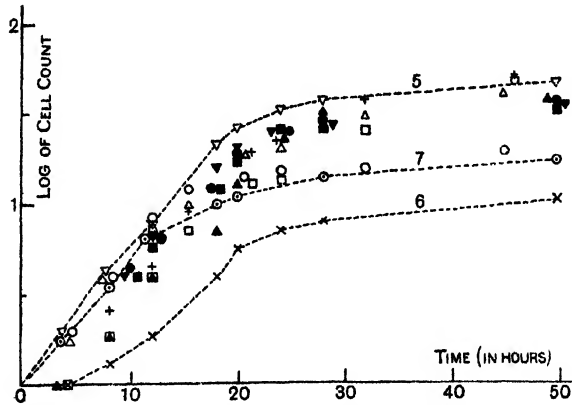


Fig. 2. Growth of washed single cells in medium composed of recrystallised salts and commercial cane sugar.

Volume (mm.<sup>3</sup>)  
40 ▲ ▼ ▣ ●  
1.4 ▽ ----- ▽  
1.3 ○ ----- ○  
1.0 × ----- ×  
0.04 □ +  
0.02 ○  
0.008 △

the microscope. If this precaution was observed the cells were counted quite easily, using a low-power objective and high-power ocular.

In Exp. 2 great difficulty was experienced in locating the single cells in the largest drops: in three cases out of the five they were not found until the third observation (10 hours).

At the end of the growth period all the cultures were examined for contamination, either by fixing and staining *in situ*, or by transferring a loopful to nutrient agar, which was then incubated at 25° for 2 days followed by a further 2 days at 37°. In the latter case smears were prepared from the agar slopes and stained and examined in the usual manner. Any infected cultures were excluded from the results.

### RESULTS.

In each experiment I have recorded on a graph the logarithm of the number of cells present at each observation. Buds were considered as fractions, computed on the basis of the relationship between their diameters and those of the cells from which they were growing. The graphs are shown in Fig. 1 (Exp. 1) and Fig. 2 (Exp. 2).

In Table I, I have compared in each experiment the total number of bacteria-free cultures which were prepared with the number of these which actually showed growth.

Table I.

Volume	Exp. 1		Exp. 2	
	Total	Growing	Total	Growing
Largest	6	4 (66 %)	5	4 (80 %)
Intermediate	—	—	4	3 (75 %)
Smallest	12	8 (66 %)	6	4 (66 %)

### DISCUSSION.

It is clear from Table I that the incidence of mortality among the cultures is fairly constant in spite of the differences in volumes. In Exp. 2 the greatest mortality occurred in the smallest volumes, possibly on account of relatively greater evaporation from the smallest drops. It seems unlikely therefore that Liebig's failure to obtain growth was due to death of the cells inoculated.

In Exp. 1 the behaviour of all the cultures up to the 20th hour was very uniform, and closely comparable to that observed in Exp. 2. After the 20th hour there was a marked retardation of growth in two of the cultures in the larger volumes (Nos. 10 and 13). At the same time one of the cultures in the smaller volumes was retarded to an even greater degree (No. 3), and in another case (No. 1) there was some retardation at first. In the latter case the drop touched the surface of the slide at the 24th hour, after which growth could no longer be observed. At the conclusion of the experiment I could only count a total of 13 cells on the slide and cover-slip, but I have omitted this observation from the graph on account of its uncertainty. With the exception

of these four cultures, uniformity of growth was observed from the 20th hour to the end of the experiment.

In Exp. 2 the largest variations in growth were observed in the three cultures in intermediate volumes (Nos. 5, 6, 7). I am inclined to attribute these variations to the relatively large degree of evaporation which must have occurred, since the drops were placed in large depression slides similar to those used for the largest drops. In this connection it is perhaps significant that the greatest variation occurred in the smallest drop (No. 6). Apart from these variations the growth of all the cultures in this experiment was strikingly uniform.

In Exp. 2, although the conditions were similar to those of Exp. 1, the final growth obtained was far less. I cannot at the moment account for this difference, but it does not appear to affect the point at issue.

Thus, not only in volumes approximately 10 times larger or smaller than those used by Liebig and Pasteur, but also in volumes comparable with those which these observers employed per yeast cell inoculated, the growth of single cells of yeast appears to be uniform. Liebig's failure to obtain growth does not therefore appear to have been due to any effect of volume *per se* on the vitality or growth of the yeast.

It would seem more probable that the absence of "bios" from Liebig's cultures affords the true explanation. For, as the following experiment shows, it is possible to obtain growth of a single washed yeast cell in a volume as large as 4.0 cc.

*Growth of single yeast cells in large volumes.*

In the following experiment I have studied the growth of single cells in volumes of 0.08, 1.0 and 4.0 cc. Hanging-droplet preparations of single washed cells in medium 2 were prepared, and the cells transferred by means of a capillary pipette as in the experiments reported above. The 0.08 cc. cultures were grown in depression-slides, the 1.0 and 4.0 cc. in test tubes of suitable size. The medium used was medium 5. Growth was determined after 48 hours at 25° by a cell-count in a Bürker counting-chamber. In the case of the 0.08 cc. cultures the cells were washed off the slide with 1.0 cc. of medium 2 into a small test tube, the medium being delivered drop by drop from a calibrated Dreyer pipette. Examination of the slide after this process showed that very little yeast remained on it.

Table II.

Exp. 3. Inoculum one cell. Thousands of cells present in each culture after 48 hours at 25° are recorded in the third column.

Volume cc.	Culture No.	Growth $\times 10^3$	Error $\times 10^3$
0.8	1	914	25
0.8	2	892	25
1.0	3	551	25
4.0	4	712	60
4.0	5	1215	80
4.0	6	922	130

The results are recorded in Table II. The distribution of cells was rather uneven, and the figures for the counts were low (about 1 for each large square of  $1/25$  mm.<sup>2</sup>). There is thus considerable error in these figures. In the last column of the table I have recorded the probable error of the counts, calculated by taking extreme values of the figures obtained for each row of 12 squares.

All the cultures in this experiment were free from contamination. With the exception of No. 3, the growth of all the cultures was approximately uniform, and there was no evidence of any inhibitory effect in the 4.0 cc. cultures. The figures are too few to justify any comparison of the effects of the different volumes, especially as the experiment has not been repeated. It is clear, however, that provided that the composition of the medium is suitable a single yeast cell can increase a millionfold in 48 hours when inoculated in a volume of medium 8,000,000,000 times its own. The growth of single cells in a medium composed of recrystallised cane sugar and salts (medium 1) has never in my experience exceeded 20-fold, and has generally been about 10-fold in 48 hours. These observations are in accord with those of Hansen [1879] and Kossowicz [1903]. The former inoculated a series of flasks with very small quantities of yeast, and, to obtain pure cultures, only employed those flasks which showed single colonies after incubation. But, as Lafar [1910] points out, cultures arising from a single cell can only be obtained from gelatin plates actually examined under the microscope, and this was the method subsequently adopted by Hansen [1883]. It cannot therefore be stated with absolute certainty that Hansen grew single cells in comparatively large volumes of fluid medium, but he certainly obtained growth from very small inocula. Kossowicz failed to observe multiplication, as judged by the naked eye, of single cells in media composed of salts and pure sugar, while large inocula ("viele Zellen") showed rich development under similar conditions. He was inclined to attribute the growth of the latter to "carry over." We must conclude therefore that failure of growth of small inoculations of yeast is due to deficiencies in the composition, and not to the volume, of the medium used. The explanation of the Liebig-Pasteur controversy as a result of differences in amount of "bios" present appears to be justified.

#### SUMMARY.

1. The growth and vitality of single cells of yeast does not appear to be affected by the volume of medium employed, provided that the composition of the medium is suitable.

2. The differences of growth observed by Pasteur and Liebig cannot be ascribed to differences of the volume of medium used per cell inoculated, but were probably due to the presence of different amounts of "bios." This conclusion, although stated in a previous communication, was not justified by the results reported at that time.

In conclusion I desire to thank Prof. Peters for his interest and advice. I am indebted to the Medical Research Council for a part time grant, and to the Trustees of the Christopher Welch Fund for the purchase of apparatus.

## REFERENCES.

- Hansen (1879). *Compt. Rend. Lab. Carlsberg*, **1**, 49, 197. Cited by Lafar (1910).  
—— (1883). *Compt. Rend. Lab. Carlsberg*, **2**, 13.  
Kossowicz (1903). *Z. Landwirtsch. Versuchs. Oesterreich*, **6**, 731.  
Lafar (1910). *Technical Mycology*, **2**, 219.  
Peskett (1925, 1). *Biochem. J.* **19**, 464.  
—— (1925, 2). *Biochem. J.* **19**, 474.

## XVII. THE STOMACH OIL OF THE FULMAR PETREL (*FULMARUS GLACIALIS*).

BY OTTO ROSENHEIM AND THOMAS ARTHUR WEBSTER.

*From the National Institute for Medical Research, Hampstead, N.W. 3.*

*(Received December 3rd, 1926.)*

IN the course of an investigation on the suggested relationship between vitamin A and the arsenious chloride colour reaction of cod-liver oils [Rosenheim and Webster, 1926, 1], it seemed desirable to examine some other oils, differing essentially in their character and origin from cod-liver oil. Our attention was drawn by Sir Walter Fletcher to a communication by Malcolm [1926], in which the stomach oil of the Australasian mutton bird or white-headed Fulmar (*Oestrelata lessoni*) was stated to be a rich source of vitamin A. Purdy [1918] had previously suggested the body-fat of these birds as a substitute for cod-liver oil, and had found it effective in the treatment of bronchitic conditions and phthisis.

We were unable to obtain mutton bird oil in this country, as it appears to be produced commercially in small amounts only in certain islands near Tasmania and New Zealand. It seemed of interest therefore to examine the stomach oil of an arctic representative of the order Tubinares, the Fulmar petrel (*Fulmarus glacialis*), which corresponds most closely to the antarctic mutton bird. These birds breed in enormous numbers in St Kilda, and have extended their breeding colonies in recent years to an extraordinary extent in the Orkney and Shetland Islands [Coward, 1926]. They are purely oceanic wanderers, and seldom, if ever, come to land, except for the purpose of breeding. When disturbed on the nest, the bird ejects, as a defensive measure, with considerable force, some of the oil stored in the stomach.

We found that the stomach oil not only gives the arsenious chloride reaction to approximately the same degree as cod-liver oil, but that its content of growth-promoting vitamin A runs parallel to the colour value. The oil also contains the calcifying vitamin D.

A chemical examination showed that the oil is not a glyceride, but a "liquid wax," containing nearly 40 % of unsaponifiable matter. The latter appears to consist mainly of unsaturated higher alcohols of the same type as those found in sperm oil. The resemblance of the Fulmar stomach oil to that contained in the head cavity of the Spermaceti Whale (*Physeter macrocephalus*, L.) is so striking as to suggest a similarity in the origin and function of these oils which will be discussed later.

## EXPERIMENTAL.

Through the kindness of Capt. S. R. Douglas we were able to examine a nearly full-grown bird, which was shot in August, at the end of the breeding season, in the island of Yell, Shetland. The bird weighed 800 g. and its wing measure was 290 mm. (adult birds = 318–330 mm.). The proventriculus was found to be completely filled with oil, which was collected by ligaturing the organ and making an incision. The oil, 50 cc. in amount, was perfectly clear, deep amber-coloured, and of a penetrating musty odour, resembling that of arctic plankton<sup>1</sup>.

The natural untreated oil was used throughout this investigation, and was protected against light and air which rapidly bleach it.

The thick layer of blubber beneath the skin yielded 72 % of a nearly colourless fluid oil. This, as well as the fat of the liver and muscular tissue, was also examined for comparison with the stomach oil. In order to avoid destruction of the vitamin during the preparation of the fats, the tissues were immediately put into 5 % KOH solution. On standing at room temperature for 24 hours, the proteins dissolved and the fats were extracted with pure ether in a separating funnel. The ether extract was washed with water, dried with anhydrous  $\text{Na}_2\text{SO}_4$  and concentrated in a current of nitrogen. The fats were finally dried *in vacuo*.

*Colour tests.*

Quantitative determinations of the blue colour given in Rosenheim and Drummond's  $\text{AsCl}_3$  test [1925], were carried out by means of Lovibond's colorimeter. The results were identical with those obtained by the more convenient modified method, introduced by Carr and Price [1926], in which a chloroform solution of  $\text{SbCl}_3$  is used instead of undiluted  $\text{AsCl}_3$ . We have, however, recently met with oils of low vitamin A content in which the modified method indicated the absence of vitamin A, whilst the results of the colorimetric "arsenic" test agreed with the biological growth test. This lack of sensitiveness in the "antimony" method is due to the dilution of both the oil and reagent and necessitates a control test with undiluted  $\text{AsCl}_3$  in all apparently negative cases.

Table I.

	Arsenic and antimony reaction			Fearon reaction		
	Mg. oil in 1 cc. reagent	Units of blue		Mg. oil in 1 cc. reagent	Units of red	
		Measured	C.L.O. = 1*		Measured	C.L.O. = 1*
Cod-liver oil	20	10.0	1.0	20	20	1.0
Fulmar stomach oil	20	8.0	0.8	20	11.0	0.55
„ subcutaneous fat	100	2.0	0.04	100	12.0	0.12
„ muscle fat	20	2.2	0.22	20	1.5	0.07
„ liver fat	0.5	16.0	64.0	20	Nil	Nil

\* C.L.O. = 1 signifies the numbers of blue or red units given by 20 mg. oil, taking cod-liver oil = 1.

<sup>1</sup> We had occasion to notice the smell of plankton also in the stomach contents of a whale (*Balaenoptera rostrata*), which had been alive for 3 days after having been stranded on the East Coast.

In Table I the results obtained with an active Newfoundland cod-liver oil are included for comparison, together with those of Fearon's [1925] colour test.

It will be seen that the Fulmar stomach oil possesses four-fifths of the colorimetric value of the standard cod-liver oil, whilst that of the subcutaneous and muscle fat is much less. On the other hand, the liver fat is 64 times as strong as cod-liver oil. The amount of this liver fat available was not sufficient for a growth test, but we found liver fats of other animals of equally high colour value, the growth-promoting power of which we are at present investigating.

Fearon's test has no relation to vitamin A, as we have previously shown, and the results are interesting only in confirming our statement that the reaction is negative in the case of liver fats of birds [Rosenheim and Webster, 1926, 1].

#### *Feeding tests.*

(1) *Vitamin A.* The technique described by Drummond, Coward and Handy [1925] was employed, in which a constant supply of vitamin D (irradiated cholesterol) is given throughout the experiment. In the course of this and similar investigations we found it convenient to correlate the dose of the oil to be tested with its colorimetric value by taking as the standard the number of blue units (Lovibond's scale) given by 10 mg. and 20 mg. of cod-liver oil in the colour test. Under the conditions adopted 20 mg. of Newfoundland oil equal 10 units of blue, and administered daily restore normal growth, *i.e.* induce a weekly increase in body-weight of 8–10 g. In the present work the Fulmar oil was administered in daily doses of 10 mg. and 20 mg., representing 4 and 8 units of blue respectively. In our experience the animal test, as at present developed, is not sensitive enough to differentiate variations of  $\pm 2$  units of blue, *i.e.* deviations of  $\pm 4$  mg. from the average dose of 20 mg. The results are represented in Fig. 1.

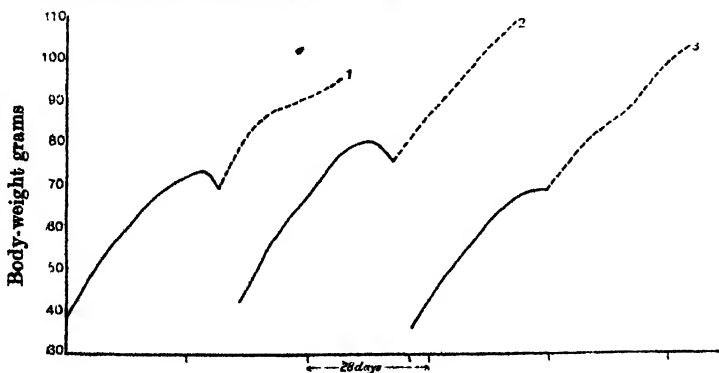


Fig. 1.

- 1 = 10 mg. Fulmar oil = 4 Blue units.
- 2 = 20 mg. " " = 8 Blue units.
- 3 = 20 mg. cod-liver oil = 10 Blue units.



It will be seen that 20 mg. (= 8 blue units) of the Fulmar stomach oil produced growth of the same order as the same quantity of the standard cod-liver oil (= 10 blue units). Xerophthalmia had developed in all cases during the preparatory period, which cleared up rapidly on administration of the oil.

The growth tests made with mutton bird oil by Malcolm [1926] are open to criticism, since a constant supply of vitamin D had not been given. The statement "that mutton bird oil is one of the richest known natural sources of vitamin A" requires modification, as a good Newfoundland cod-liver oil induces similar growth increments in the doses given by Malcolm.

(2) *Vitamin D*. The technique previously described [Rosenheim and Webster, 1926, 2] was used. A daily dose of 20 mg. gave partial protection, whilst 40 mg. completely protected the animals against rickets. The presence of vitamin D in mutton bird oil has up till now not been demonstrated.

#### *Chemical examination.*

The untreated stomach oil of the Fulmar is a clear, deep orange-red, non-drying oil which begins to solidify when cooled to 8–9°, becomes waxy at 5° and sets to a translucent solid at 0°. Table II contains the constants of the oil, determined by the standard methods, together with those of mutton bird oil<sup>1</sup> [Carter, 1921], a genuine sperm oil [Dunlop, 1908], and an average cod-liver oil.

Table II.

	Fulmar oil	Mutton bird oil	Sperm oil	Cod-liver oil
Specific gravity	0.884	0.884	0.880	0.925
Saponification value	122	120	122	185
Iodine value (Wijs)	118	130	70	168
Higher alcohols	37.7 %	38.4 %	41.2 %	0.5–1.5 %
M.P. of ditto	31.5°–32.5°	31°–31.5°	31.5°–32.5°	?
Free fatty acid	1.6	2.2	1.4	0.3–1.5
Cold test	8°–9°	6°	7°–9.5°	Variable

These figures justify the conclusion that the composition of the stomach oil of these representatives of the arctic and antarctic petrels is practically identical. Both oils differ fundamentally from cod-liver oil in their low specific gravity and their high percentage of unsaponifiable substances (alcohols) which characterise them as "liquid waxes." On the other hand, they both resemble sperm oil, their higher iodine value being due to the nature of the unsaturated fatty acids they contain.

*The fatty acids* were fluid at room temperature. In the elaidin test they solidified only partially, thus indicating the presence of a certain amount of oleic acid. Their mean molecular weight was 292 and the iodine value 156. The high iodine value points to the presence of highly unsaturated fatty acids and the bromination test was therefore carried out.

<sup>1</sup> The earlier analyses of mutton bird oil [Smith, 1911; Evers and Foster, 1920] are in general agreement with that by Carter [1921].

1.134 g. of the fatty acids gave 0.238 g. = 20.9 % of ether-insoluble bromides. The white substance did not melt when heated in a capillary to 200° and began to darken at 240°, turning black without fusing at 250°. The product, being insoluble in organic solvents, was purified by extraction at the boiling-point with ether and benzene, in which it was practically insoluble. The behaviour on heating remained unchanged, thus excluding the presence of the hexabromide of linolenic acid (M.P. 175°).

*Analysis:* 0.0620 g. gave 0.1038 g. AgBr. Found 71.3 % Br.

Calculated for  $C_{22}H_{34}O_2Br_{10}$ : 70.9 %

„ „  $C_{20}H_{32}O_2Br_8$ : 67.8 %.

The substance is therefore the decabromide of clupanodonic acid, which has been found by Tsujimoto [1922] as a characteristic constituent of sardine oil and other fish oils. The amount of ether-insoluble bromides in sperm oil is only 1.1–2.3 % [Dunlop, 1908], and this fact explains the difference in the iodine value of Fulmar and sperm oil.

The fatty acids give Fearon's test, which is ascribed by Rosenheim and Webster [1926, 3] to aldehydic oxidation products of clupanodonic acid.

*The alcohols.* The unsaponifiable fraction of the oil is a deep ruby-red wax, which rapidly bleaches on exposure to light and air. The  $AsCl_3$  reaction is given in great intensity by freshly prepared specimens. Liebermann's cholesterol reaction is positive and Fearon's reaction negative, as in the unsaponifiable fraction of cod-liver oil. The alcohols were recrystallised from light petroleum, filtered at a low temperature and were thus obtained as a pale yellow wax, melting at 32°, easily soluble in organic solvents. Their iodine value is 54, and on bromination a small amount of a white bromide (cholesterol bromide?) is obtained. A quantitative estimation, by means of digitonin, gave 2.4 % cholesterol, showing that the bulk of the alcohols is unsaturated. On acetylation with acetic anhydride the acetates were obtained as oils, which solidified at 0° and melted at 5°. The acetates formed a clear solution in acetic anhydride, indicating the absence of any considerable amount of hydrocarbons. We were unable to obtain any direct evidence for the presence of cetyl alcohol, which is assumed to constitute the bulk of the alcohols of mutton bird oil [Carter, 1921]. In their general character the alcohols of the Fulmar oil resemble the unsaturated alcohols of unknown constitution, which have been obtained from sperm oil by Lewkowitsch [1892]. Recently oleic alcohol has been isolated from the unsaponifiable fraction of arctic sperm oil by Tsujimoto [1925].

The similarity in composition of the two oils is further exemplified by the fact that we were able to isolate only 1.4 % of glycerol from Fulmar oil, and that sperm oil also yields not more than 1.3–2.5 % glycerol [Dunlop, 1908].

*The lipochromes.* The solution of the pigments in light petroleum, obtained on recrystallisation of the alcohols (see above) was subjected to the Kraus phase-test [Willstätter and Stoll, 1913]. The pigment was found to be entirely epiphasic, thus showing it to be related to, or identical with, carotene, and

proving the absence of xanthophyll. This was confirmed by the chromatographic analysis according to Tswett, in which the light petroleum solution passed completely through the chalk column without showing any xanthophyll layers. Spectroscopic examination also indicated the presence of carotene, although the second and third absorption bands were partially obscured by general absorption.

The pigment is easily absorbed by charcoal (norit) from an ethereal solution of the oil. The colourless filtrate, after removal of the ether, gave quantitatively the same amount of blue colour with  $\text{AsCl}_3$  as the original oil. The presence of the lipochrome therefore does not interfere with the colour test.

*Possible function of the stomach oil.*

We were unable to find any previous reference in the literature throwing light on the presence of the large amounts of oil in the stomach of the Fulmar petrel, except that it appears to be peculiar to all the members of the order Tubinares [Challenger Reports, 1882]. Since the oil, like other waxes, is resistant to hydrolysis by lipoclastic enzymes, it might be assumed to represent the indigestible residue of fatty foodstuffs. Such an assumption has indeed been made by Carter and Malcolm [1926], who were unable, however, to find any evidence for the presence of similar waxes or their alcohols in the fats of fishes, or any form of marine life, which might serve as food for these birds.

The resemblance of this fluid wax in its chemical character to that secreted by the "preen" glands of terrestrial birds suggests a different explanation. The rump gland wax of geese and ducks contains, like the stomach oil of petrels, about 40 % of alcohols, in which the presence of octadecyl alcohol has been definitely established by Röhmann [1904]. This wax has been shown by Röhmann to be a true secretory product and its obvious use to the bird in preening is to protect the feathers against the action of water, for which it is better suited than the easily saponifiable fats.

We would suggest that the stomach oil of the petrels has a similar origin and fulfils a similar function in these marine birds, which owing to their environmental conditions require a larger supply of preening material than terrestrial birds. Such a suggestion is strengthened by the presence of a distinctive structural feature in the order Tubinares, i.e. "their prominent tubular nostrils and their bills which consist of several horny pieces, separated by deep grooves" [Godman, 1910]. Through these structures the oil may be distributed over the beak, thus facilitating the preening operation when the bird is resting on the sea<sup>1</sup>. Another peculiarity of the oil, shared by sperm oil, is its low viscosity, which according to our determination is 0.216 at body temperature (36°). This characteristic feature distinguishes it from animal

<sup>1</sup> It might be mentioned that the petrels are said to eject the oil "through their nostrils" when disturbed. Certain statements in the literature lend support to the suggestion that the secretory gland may have to be searched for near the nasal cavity, the proventriculus serving merely as a store for the secretion.

fats and renders easy the transference of the oil from the proventriculus to the beak and aids in its distribution over the feathers. The presence of unsaturated fatty acids and alcohols in the wax helps to keep it fluid at relatively low temperatures, a property especially useful to a bird whose life is spent in the arctic regions.

The above view is rendered still more likely when we consider the analogous composition and function of similar waxes in certain aquatic mammals, the cetaceans. The liquid wax, sperm oil, present in the head cavity of many whales is practically of the same composition as the Fulmar stomach oil (see above), whilst that of dolphins and porpoises appears to form an intermediate link between liquid waxes and blubber oils [Lewkowitsch, 1921]. In the whale a canal running along the whole length of the body to the tail communicates with the head cavity and, probably in a way at present not properly understood, enables the secretion to reach and to spread over the surface of the skin, thus protecting the animal against the action of the sea water [Röhmman, 1908].

This suggested explanation for the function of the stomach oil does not account for the presence in it of vitamins. It may be pointed out, however, that the oil which we examined came from a juvenile bird which had not yet left the nest. The food received from the parents would probably supply more than the necessary amount of fat-soluble vitamins and thus explain their presence in the stomach oil. The examination of the stomach contents of a non-breeding adult bird would be of interest in this respect, and we intend to examine this and other questions during the next breeding season.

#### SUMMARY.

1. The stomach oil of the Fulmar petrel (*Fulmarus glacialis*) has been examined chemically and biologically. It gives the arsenious chloride reaction approximately to the same degree as cod-liver oil, and its content of vitamin A runs parallel to the colour value. The oil also contains vitamin D.
2. The oil is not a glyceride, but a liquid wax of similar composition to sperm oil.
3. An explanation for the origin and the function of the oil is suggested.

#### REFERENCES.

- Carr and Price (1926). *Biochem. J.* **20**, 497.  
Carter (1921). *J. Soc. Chem. Ind.* **40**, 220.  
Carter and Malcolm (1926). *Trans. New Zealand Inst.* **56**, 647.  
Challenger Reports (1882), **4**, Pt XI, *Anatomy of the Petrels*.  
Coward (1926). *The Birds of the British Isles* (London).  
Drummond, Coward and Handy (1925). *Biochem. J.* **19**, 1068.  
Dunlop (1908). *J. Soc. Chem. Ind.* **27**, 63.  
Evers and Foster (1920). *Pharm. J.* **51**, 100.  
Fearon (1925). *Biochem. J.* **19**, 888.  
Godman (1910). *Monograph of the Petrels* (London).  
Lewkowitsch (1882). *J. Soc. Chem. Ind.* **11**, 134.

- Lewkowitsch (1921). *Chem. Techn. Oils, Fats and Waxes* (London).
- Malcolm (1926). *Trans. New Zealand Inst.* **56**, 650.
- Purdy (1918). *Brit. Med. J.* **i**, 174.
- Röhmman (1904). *Hofmeister's Beitr.* **5**, 110.
- (1908). *Biochemie* (Berlin)
- Rosenheim and Drummond (1925). *Biochem. J.* **19**, 753.
- Rosenheim and Webster (1926, 1). *Lancet*, **ii**, 806.
- — (1926, 2). *Biochem. J.* **20**, 537.
- — (1926, 3). *Biochem. J.* **20**, 1342.
- Smith (1911). *J. Soc. Chem. Ind.* **30**, 405.
- Taujimoto (1922). *Chem. Umschau*, **29**, 261.
- (1925). *Chem. Umschau*, **32**, 125.
- Willstätter and Stoll (1913). *Untersuch. über Chlorophyll* (Berlin).

# XVIII. STUDIES ON THE KINETICS OF HAEMOLYTIC SYSTEMS.

## III. TIME-DILUTION CURVES AND ZONES OF ACTION.

By ERIC PONDER.

*From the Department of Physiology, Edinburgh University.*

*(Received November 23rd, 1926.)*

THIS paper, which is intended to amplify one already published [Ponder, 1926, 2] is concerned with the expression

$$\kappa t = \frac{1}{S - \psi s} \log \frac{c}{c - \psi x - x} \dots\dots\dots(1),$$

which, when applied to a population of cells whose resistances are distributed according to a normal frequency curve, describes in a very satisfactory manner the action of the simple haemolysins<sup>1</sup>. In the expression,  $c$  is the concentration of lysin acting on the cells,  $x$  the quantity used up in the reaction with the cells,  $\kappa$  a velocity constant, and  $t$  the time taken for the lysin to bring about any specified degree of lysis;  $S$  is a term relating to the surface of the suspension, while  $\psi$  denotes a probability which can vary between 0 and 1. The derivation of the expression is discussed in the paper quoted.

This paper falls into two parts. In the first part we shall examine the extent to which the expression (1) describes the time-dilution curves of various lysins, and in the second part we shall find values for  $\psi$ , and thence values for the size of the hypothetical "zone of action" surrounding the cells of the particular haemolytic systems considered, within which the reaction between the cells and the lysin may be imagined to proceed.

### 1. THE TIME-DILUTION CURVES.

The time-dilution curve for any haemolysin is obtained by plotting the dilution of lysin against the time which it takes to bring about complete haemolysis of a certain number of cells. The technique is fully given in a previous paper [Ponder, 1926, 1] and papers quoted therein. In all the cases given below the suspension used was prepared by suspending the thrice washed cells from 1 cc. of blood in 20 cc. of 0.85 % NaCl. Of this suspension, 0.4 cc. was added to 1.6 cc. of a solution of lysin of such a strength as to give a known dilution of lysin after the mixture. In this way the dilution of lysin

<sup>1</sup> In the paper quoted, the final  $x$  in the denominator has been dropped by a printer's error

upon which the calculations are based is that present in the fluid in which the cells are suspended at the beginning of the haemolytic reaction. The lysins were all dissolved in 0.85 % NaCl.

We are here concerned with the extent to which equation (1) describes these time-dilution curves, and shall therefore tabulate experimental and calculated results. In these tables, the dilution of lysin will be replaced by a concentration—the number of milligrams of lysin present in the tube containing 2 cc. of fluid. This is for convenience in calculation only.

In using equation (1) for the calculation of the time-dilution curves, we may simplify it by putting  $S$  equal to unity, and  $\psi$  equal to zero. The expression then reduces to the well-known expression for a monomolecular reaction

$$\kappa t = \log \frac{c}{c-x}.$$

It cannot be too strongly emphasised, however, that this simplification is permissible only because of the special use to which the expression is to be put: in general the simplification cannot be made.

Concentration mg.	$t$ experi- mental mins.	$t$ calcu- lated mins.	Concen- tration mg.	$t$ experi- mental mins.	$t$ calcu- lated mins.
1. Ammonia and human erythrocytes at 25°			2. Sodium stearate and human erythrocytes at 25°		
		$x=15, \kappa=0.25$			$x=0.32, \kappa=0.20$
64	0.9	1.0	2.0	0.9	0.85
34	2.1	2.2	1.0	2.0	1.9
23	4.1	4.25	0.5	4.6	5.1
17	9.0	8.5	0.4	8.0	8.0
			0.3	16.0	15.0
3. Sodium palmitate and human erythrocytes at 25°			4. Sodium oleate and human erythrocytes at 25°		
		$x=0.25, \kappa=0.25$			$x=0.048, \kappa=0.166$
2.0	0.5	0.56	0.2	1.5	1.50
1.0	1.0	1.0	0.1	3.6	3.8
0.5	2.6	2.8	0.06	8.0	7.8
0.4	4.2	4.0	0.05	20.0	19.2
0.3	7.7	7.2			
0.285	9.0	8.4			
5. Sodium taurocholate and human erythrocytes at 25°			6. Saponin and human erythrocytes at 25°		
		$x=0.33, \kappa=0.178$			$x=0.028, \kappa=0.303$
2.0	0.8	0.9	0.2	0.4	0.49
1.0	1.9	2.1	0.1	1.0	1.1
0.5	5.8	6.0	0.05	2.7	2.7
0.4	9.7	9.7	0.04	4.0	4.0
			0.03	6.2	6.1
			0.0285	13.0	12.8

These results are sufficient to show that the degree of exactness with which the times for complete haemolysis can be calculated from equation (1) is considerable. The nature of the lysins, too, is sufficiently varied to show that the expression is one of general applicability. This list might be greatly extended by including the action of each of these lysins on the cells of various

animals; it has been thought unnecessary to do this, for we have shown in the first paper of this series that the relation of the action of saponin on human cells to the action on the cells of any of the mammalia is such that the resistance of the two systems can be expressed by a constant  $R$ . Reference to another publication [Ponder, 1926, 2] will show that the appearance of this constant indicates that, if the action of saponin can be described by an expression such as (1), the action on the cells of the sheep, dog, cat, or whatever animal is selected, must be described by an expression of the same type.

We might extend the list by including other lysins. Those given above are the common ones, and the action of the rarer lysins must be left for future investigation.

## 2. THE ZONE OF ACTION.

In a haemolytic system such as one containing red cells and saponin, it is plain that the reaction which results in lysis cannot occur generally throughout the system, but only in the neighbourhood of the cell surfaces. The reaction is therefore discontinuous, and in order to take the idea of discontinuity into account, we use the idea that each cell is surrounded by a zone within which the reaction can be localised, and to which the term "zone of action" is applied.

It is shown in the paper quoted above [Ponder, 1926, 2] that the size of the zone of action may be approximately obtained in a very simple way. We take the most dilute suspension of red cells with which it is possible to work, plot its time-dilution curve, and find  $c_{1\infty}$ , the concentration of lysin which corresponds to the asymptote. A suspension of a known cell content, some four times greater than that of the very dilute suspension, is then prepared, its time-dilution curve plotted, and  $c_{2\infty}$ , the concentration of lysin corresponding to the asymptote found.

Then we have  $\frac{c_{2\infty}}{c_{1\infty}} - 1 = \psi \dots\dots\dots(2).$

But in the nomenclature of another paper [Ponder, 1926, 1], the ratio  $c_{2\infty}/c_{1\infty}$  is equal to  $R$ , the resistance constant of the second suspension as compared with the first, and this constant has the same value for all values of  $t$ . In order to avoid confusion in what follows, we shall call the resistance constant, when it is obtained from two suspensions such as the above for the purpose of working out the dimensions of the zone of action, not  $R$ , but  $R_z$ . So we have

$$R_z - 1 = \psi,$$

$$\text{or } R_z - 1 = v/V \dots\dots\dots(3),$$

where  $v$  is the volume of all the zones of action added together, and  $V$  the volume of the whole haemolytic system.

Call  $v'$  the volume of a single zone of action, and we get

$$\frac{V(R_z - 1)}{N} = v' \dots\dots\dots(4),$$

where  $N$  is the number of cells in the haemolytic system which contains the greater number of cells.



If  $\rho$  is the radius of the zone of action measured from the surface of the cell outwards (see Fig. 1),

$$\rho = \sqrt[3]{\frac{3V(R_z - 1)}{4\pi N}} - r \quad \dots\dots\dots(5),$$

where  $r$  is the radius of the cell in the spherical or Goughian form.

The determination of the dimensions of the hypothetical zone of action is therefore an easy matter, for all that has to be done is to determine  $R_z$  for a suspension of known cell content and of arbitrary strength as compared with the most dilute suspension with which it is possible to work.

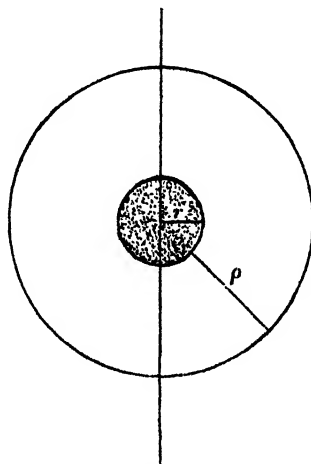


Fig. 1.

Diagram of the zone of action. The cell of radius  $r$  is surrounded by the zone, extending outwards for a distance  $\rho$ . The figure rotated about the vertical line gives a solid of volume  $v'$ . The volume of the sphere representing the cell is  $v''$ , and the volume of the spherical zone surrounding it is  $Z$ .

Suitable strengths of the suspensions are as follows: (a) a suspension containing in 20 cc. of 0.85 % NaCl the thrice washed cells from 1 cc. of blood; (b) a suspension containing one-fourth of the cells in suspension (a). With a few exceptions, it is impossible to obtain good end-points with suspensions less concentrated than (b); in any case, the zones of action appear in such suspensions to be completely separated, which is the essential point.

When two suspensions such as these are used,  $R_z$  will always be found, irrespective of the type of cell dealt with, to be less than 2. This is, of course, essential, for if  $R_z$  exceeds 2, the zones of action completely overlap, and the limits within which the experimental procedure is possible are overstepped.

We shall illustrate the method by an example.

Lysis of sheep cells by saponin. The strengths of the two suspensions were as in (a) and (b) above.

For suspension (a) the asymptote of the time-dilution curve at 25° was found to correspond to a concentration of lysin of 0.210 mg.

For suspension (b) the asymptote corresponded to a lysin concentration of 0.130 mg.

Dividing the one figure by the other we get  $R_s = 1.63$ , and so

$$(R_s - 1) = 0.63.$$

This figure is less than 1, as it ought to be. The number of cells in the system was  $2 \cdot (10^8)$ , the volume of the system 2 cc., and the radius of the sheep cell, in the spherical form,  $2\mu$ .

Applying equation (5) with these values inserted, we find that the value of  $\rho$  works out as  $9.5\mu$ . The zone of action accordingly extends, in the case of this haemolytic system, from the cell surface outwards for a distance of  $9.5\mu$ .

We now proceed to apply this method to the cells of various animals, with a view to determining whether the radius of the zone of action is constant, or whether it varies with the type of cell to be haemolysed.

All the experiments were carried out at  $25^\circ$ . The results are shown in the following table, the types of cell being arranged according to increasing size of the zone of action:

Animal	$R_s$	$N \times (10^{-8})$	$r, \mu$	$\rho, \mu$
Rabbit	1.07	1.0	2.5	4.5
Man	1.14	1.0	3.0	5.8
Dog	1.18	1.4	2.5	6.0
Cat	1.23	1.2	2.3	7.4
Ox	1.59	1.6	2.3	9.0
Sheep	1.63	2.0	2.0	9.5

A glance at the table will show the remarkable nature of the result, for in the table we have the cells arranged in Ryvosh's series [Ryvosh, 1907; Ponder, 1927]. The order of the cells arranged according to the size of the zone of action is thus the same as the order of the cells arranged according to resistance to saponin. The most resistant type of cell—that of the sheep—has the largest zone of action, the least resistant type—that of the rabbit—the smallest zone of action.

Turning to the idea of the zone of action as a zone within which there exists a concentration gradient of lysin, and regarding its boundary as a point where the slope of this concentration gradient becomes negligible, we see at once the meaning of this important result. In the case of a cell which is very resistant to saponin, such as that of the sheep or ox, a large amount of saponin requires to be used up in order to bring about lysis. As a result, the zone of action is large. If, on the other hand, we have a cell such as that of man or of the rabbit, little saponin needs to be used up to bring about lysis, and the zone of action will be comparatively small. That the size of the zone of action must be related to the resistance of the particular type of cell to the lysin of the system is therefore plain from first principles, and no result other than that of the above table could be expected.

We may, however, go further, and try to find the exact relation between the size of this zone of action and the resistance presented by the particular type of cell. As has been seen in previous papers, the resistance of a particular haemolytic system, compared with that of another haemolytic system

chosen as a standard, may be expressed as the value of a constant  $R$ . This constant is obtained by dividing the concentration of lysin which corresponds to the asymptote of the time-dilution curve at  $25^\circ$  (or any other convenient temperature), for a haemolytic system containing saponin and the particular type of cell whose resistance is to be determined, by the concentration of lysin corresponding to the asymptote of the time-dilution curve at  $25^\circ$  for a haemolytic system containing the cells to be used as a standard, together with saponin. Usually it is convenient to use human cells as the standard type. For the detail of this method a previous paper [Ponder, 1926, 1] should be consulted.

In using this method of comparing resistance of cells, it is to be remembered that  $R$  refers to an entire haemolytic system. If we wish to use values of  $R$  to show the resistance of the individual cell to the lysin—or, what is the same thing, to show the amount of lysin combining with the individual cell—we must be very careful that the haemolytic systems from which the values of  $R$  are obtained are in every respect comparable. They must be comparable as regards the state of the zones of action in the system; it is no use comparing two systems in one of which the zones overlap and in the other of which they are separate. Further, if  $R$  is to indicate the amount of lysin used up by the single cell, the systems must have the same cell content. We must therefore determine values of  $R$  for systems in which the zones of action are all separate, and in which the chance of a cell entering the zone of action of another cell is so small as to approach zero. Having done this, the systems have to be reduced to the same red cell content. This point deserves the most careful consideration. A value of  $R$  determined in this way may be properly compared with figures for the dimensions of the zone of action.

With these values of  $R/N$  we shall tabulate values of the volume of the zone of action. Call this volume  $Z$ ; it is obtained from  $(v' - v'')$ , where  $v'$  is given by equation (4), and where  $v''$  is the volume of the cell supposed to occupy the centre of the zone:

Animal	$R/N \times (10^6)$	$v', \mu^3$	$v'', \mu^3$	$Z, \mu^3$
Rabbit	0.46	1400	65	1335
Man	0.88	2800	110	2690
Dog	0.71	2540	65	2485
Cat	1.4	3630	51	3779
Ox	2.5	7275	51	7224
Sheep	2.15	6300	33	6267

If the values of  $R/N$  be plotted against the corresponding values of  $Z$ , a fairly good straight line will be obtained. This line is shown in Fig. 2. The points lie along the line in a very satisfactory manner considering the amount of calculation necessary in their derivation.

The equation of this line is

$$Z = \frac{3 \cdot (10^{11}) \cdot R}{N} \dots\dots\dots (6),$$

in which  $R$  refers to suspensions in which the zones of action are completely separated, and in which  $Z$  is given in  $\mu^3$ .

We may compare the experimental results with those given by this expression, and summarise the results in the following table:

Animal	$Z$ , exper.	$Z$ , calc.
Rabbit	1335	1380
Man	2690	2640
Dog	2485	2130
Cat	3779	4200
Ox	7224	7500
Sheep	6267	6750

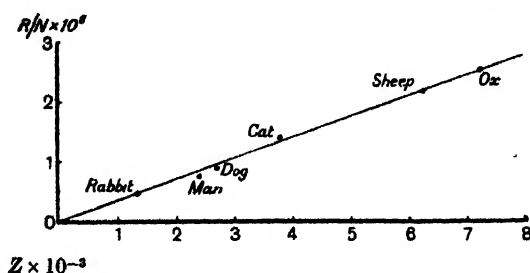


Fig. 2.

Bearing in mind that  $Z$  is in  $\mu^3$ , the correspondence is excellent.

The volume of the zone of action is thus proportional to the amount of lysin used up by the cell in the haemolytic reaction. This result, it will be observed, is by hypothesis the only possible one, for we have defined the zone of action as containing the lysin which reacts with the cell. While, however, the result could have been obtained from the original conditions as a deduction, we have here obtained it in a somewhat different way, by the consideration of Ryvosh's series, and have thus linked up this series with the fundamental equation (equation (1)), with the conception of the zone of action, and with the resistance constants for the different types of cell. The fact that so many different methods of attack yield the same result is the strongest evidence of the correctness of all the underlying assumptions, and of the applicability of the fundamental equation to the kinetics of a simple haemolytic system in which saponin is the lysin.

It will be appreciated that there is much room for the extension of these methods to other haemolytic systems. These extensions we propose to deal with later.

Finally, it is instructive to represent the conception of the zone of action, regarded as a concentration gradient, in a graphical form. This is done in Fig. 3. The cell, of radius  $r$ , is represented by a thick line. At its surface the concentration of lysin is taken to be zero, and in the concentration gradient of radius  $\rho$  this concentration is supposed to rise to a value given by  $R/N$  as measured in equation (6). Between the values of  $R/N$  and zero the concentration falls according to some curve which we cannot at present determine, so we represent it merely by a straight line. In fact, it is probably a curve of exponential character. These lines are shown in the diagrams, one of which refers to the cell of the rabbit, while the other refers to the cell of the sheep.

The fall in the case of the sheep cell is very marked, and the concentration gradient very steep, as we should expect from the great resistance of this type of cell to saponin; in the case of the rabbit cell, the concentration gradient is much less steep, corresponding to the relatively low resistance of this type of cell.

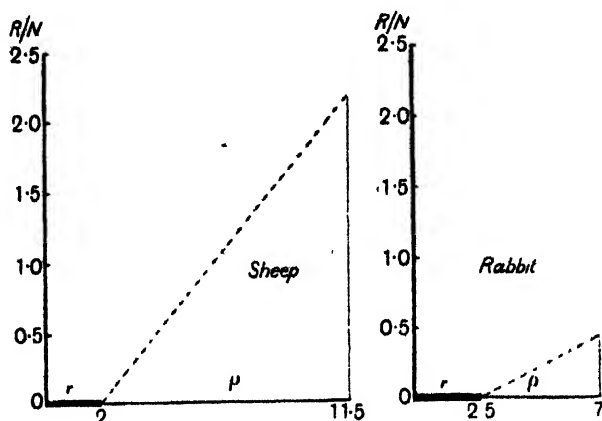


Fig. 3.

Although these figures are drawn to scale, it will be understood that they represent only in a very approximate way the conditions present, and that they are to serve no purpose except to give a general idea of the state of affairs at the cell surfaces.

#### SUMMARY.

1. A series of tables comparing experimental and calculated results for different haemolytic systems is given, together with the expression used for obtaining the calculated results.

2. A method for determining the dimensions of the zone of action round the cells of a haemolytic system is described. The radii of the zones are determined for the cells of different animals, and it is shown that the cells, arranged in order of increasing size of zone of action, fall into Ryvosh's series.

3. It is shown that the volume of the zone of action is proportional to the resistance of the cell to the lysis, a relation which follows from theoretical considerations.

This research was carried out under a grant from the Government Grants Committee of the Royal Society.

#### REFERENCES.

- Ponder (1926, 1). *Proc. Roy. Soc. Lond. B*, **99**, 461.  
 — (1926, 2). *Proc. Roy. Soc. Lond. B*, **100**, 199.  
 — (1927). *Biochem. J.* **21**, 56.  
 Ryvosh (1907). *Pflüger's Arch.* **116**, 229.

# XIX. THE RELATION OF CHOLESTEROL TO VITAMIN D.

BY OTTO ROSENHEIM AND THOMAS ARTHUR WEBSTER.

*From the National Institute for Medical Research, Hampstead, N.W. 3.*

*(Received December 20th, 1926.)*

IN continuation of our work on the molecular groupings essential for the successful activation of sterols by ultraviolet light [Rosenheim and Webster, 1926] we had the privilege of collaborating with Prof. Windaus of Göttingen in the examination of various isomers of cholesterol, recently prepared by him, and of other sterols. The details of this work which gave entirely negative results [see also Hess and Windaus, 1926] we hope to publish shortly in conjunction with Prof. Windaus.

Our failure to find any derivatives of sterols which were either active by themselves or could be rendered antirachitic by irradiation, led us to a reconsideration of the problem. The following facts emerge from previous work.

1. At least 99.9 % cholesterol is recovered unchanged when the active substance is separated from cholesterol, irradiated in a nitrogen atmosphere, by precipitation with digitonin. Further, no appreciable difference in activity is observed when the time of irradiation is varied from only a few minutes to many hours. Apparently only a small amount of cholesterol can be converted into the active condition.

2. Changes produced in the cholesterol molecule by oxidation of the hydroxyl group, or replacement of this group by chlorine, or its complete removal, deprive the derivatives so formed (cholestenone, cholesteryl chloride, cholestene) of the power to be rendered antirachitic by ultra-violet light. The presence of the OH group appears therefore essential, but the fact that the esters of cholesterol (acetate, palmitate) can be activated is contrary to this conclusion, unless the assumption is made that the esters are slightly hydrolysed during irradiation.

3. Phytosterols prepared from a sample of corn oil, which had been kept many years, cannot be activated [Steenbock and Black, 1925]. Further, another phytosterol, stigmasterol, prepared by Windaus and Hauth [1906] by way of the tetrabromide, has been found by Windaus to be incapable of being activated, a fact which we were able to confirm.

4. Allo-cholesterol, a new isomer of cholesterol recently discovered by Prof. Windaus, cannot be activated although it easily changes into cholesterol. We have recently been informed by Prof. Windaus that the actual specimen of allo-cholesterol examined by us contained some free cholesterol.

Arising out of these considerations, it seemed to us essential to investigate the question, how far the purity of the cholesterol employed is related to its capacity for activation by ultra-violet light. Although we ourselves and others had used specimens of cholesterol which had been purified with special care by the usual methods (saponification and recrystallisation, etc.) we decided to purify it still further by a chemical method. For this purpose a many times recrystallised specimen of cholesterol (m.p.  $148-9^{\circ}$ ) was converted into the dibromide, and the latter again reduced to cholesterol by means of sodium amalgam in the presence of acetic acid. The melting point of the purified substance remained unchanged ( $148-9^{\circ}$ ) and it gave the usual colour reactions with great intensity.

This product was irradiated for 1 hour and tested biologically by the methods previously used [Rosenheim and Webster, 1926].

Details of the animal experiments are given in the following table:

Rat	Daily dose mg.	Inorganic blood phosphate mg. %	X ray result
1	2	2.3	Rickets
2	4	2.4	"
3	8	2.4	"
4	Control	2.2	"
5	"	2.3	"

The result of this series of experiments is striking, especially when we consider that the original cholesterol preparation, when irradiated, prevented rickets in rats even when administered in such small doses as 0.5 mg. *per diem* [Rosenheim and Webster, 1925]. Purification by way of the dibromide completely deprived this cholesterol, which would previously have been considered as "chemically pure," of its power to become antirachitic by irradiation with ultra-violet light.

This observation throws new light on the photo-chemical formation of vitamin D. It is evident that the precursor of vitamin D is not cholesterol itself, but a substance which is associated with and follows "chemically pure" cholesterol in all its stages of purification by the usual methods (esterification, saponification, recrystallisation).

The possibility that the absorption spectrum of cholesterol may be due to a small amount of an impurity had indeed already been suggested by Schlutz and Morse [1925] in their careful study of the absorption spectra of cholesterol. The precursor of vitamin D<sup>1</sup> need not necessarily be an extraneous impurity of ordinary cholesterol. When we consider the ease with which sterols form stable complex additive compounds such as the "phytosterol" of calabar beans [Windaus and Hauth, 1906] and the complex of  $\beta$ -cholestanol with pseudocoprosterol [Windaus and Uibrig, 1915], the possibility must be kept

<sup>1</sup> In our first communication (*J. Soc. Chem. Ind.* 45, 932) we proposed the name "Vita-sterol" for the precursor of vitamin D, but as this term might lead to confusion we have adopted the more expressive name "Provitamin," suggested by Prof. Windaus, for the parent substance of vitamin D.

in mind that the provitamin may be a substance allied to cholesterol in character. The amount present in ordinary "pure" cholesterol may be judged from the yield of active substance obtained by us [Rosenheim and Webster, 1926] in the digitonin experiment, to be of the order of 1<sup>1</sup>/<sub>10</sub> %. Preliminary experiments seem to justify the statement that provitamin D is precipitated by digitonin and that its separation from cholesterol may be possible by fractional precipitation or extraction of the digitonin complex.

It is obvious that the nature of this unidentified substance, its relation to cholesterol, and its separation from it can now be investigated by experimental means.

In view of the spectrographic work of Hess and Weinstock [1925], and of Schlutz and Morse [1925] it is suggestive that, according to information kindly supplied by Prof. Windaus, a specimen of cholesterol, prepared by him at our suggestion by way of the dibromide, no longer showed the absorption spectrum in the ultra-violet region characteristic of ordinary "pure" cholesterol. This specimen had been converted into the dibromide, which was recrystallised twice and then reduced with zinc dust in the presence of acetic acid, the whole series of operations being repeated.

It is interesting to note that Heilbron, Kamm and Morton [1926] as the result of an analysis of the ultra-violet absorption spectra of cholesterol make the suggestion that cholesterol may not be the precursor of vitamin D, but that the activation of ordinary cholesterol may be due to the presence in it of traces of an unknown substance<sup>1</sup>.

#### REFERENCES.

- Heilbron, Kamm and Morton (1926). *J. Soc. Chem. Ind.* **45**, 932; see *Biochem. J.* (1927), **21**, 78.  
 Hess and Weinstock (1925). *J. Biol. Chem.* **64**, 193.  
 Hess and Windaus (1926). *Proc. Soc. Exp. Biol. Med.* **24**, 171.  
 Rosenheim and Webster (1925). *Lancet*, i, 1025.  
 ——— (1926). *Biochem. J.* **20**, 537.  
 Schlutz and Morse (1925). *Amer. J. Dis. Child.* **30**, 199.  
 Steenbock and Black (1925). *J. Biol. Chem.* **64**, 263.  
 Windaus and Hauth (1906). *Ber. deutsch. chem. Ges.* **39**, 4378.  
 Windaus and Uibrig (1915). *Ber. deutsch. chem. Ges.* **48**, 857.

<sup>1</sup> Our biological test of two preparations of these authors is referred to in their paper in this number of the Journal.

[Addendum: Jan. 31st, 1927.] Since the above communication went to Press, it has been found that ergosterol, or a sterol of similar constitution, is the parent substance of vitamin D. A preliminary communication by ourselves on this work is in the Press (see *Lancet*, Feb. 4th, 1927) and a similar one will be made in Germany by Prof. Windaus.



## XX. STUDIES ON THE CHOLESTEROL CONTENT OF NORMAL HUMAN PLASMA. PART I.

BY JOHN ADDYMAN GARDNER AND HUGH GAINSBOROUGH.

*From the Biochemical Department, St George's Hospital, London.*

*(Report to the Medical Research Council.)*

*(Received November 27th, 1926.)*

DURING the past 20 years a very large number of observations have been published on the total cholesterol content of blood or of serum from pathological cases. As a result, the blood picture is described, in any one case, as a hypercholesterolaemia or a hypocholesterolaemia, in comparison to a so-called normal value of total cholesterol in human blood. It is, however, to be noted that relatively few determinations have been made on normal subjects, and that values of the normal, obtained from these sparse data—not too concordant in the different researches—have been quoted from author to author.

It is generally agreed that in certain forms of nephritis, irregularly in diabetes mellitus and also in the later months of pregnancy, a hypercholesterolaemia is found, and that in infective conditions a hypocholesterolaemia occurs. As regards other diseases it is impossible in the present state of our knowledge of the physiological limits of cholesterol content of the blood to decide if there is any constant alteration in the level of this value. Denis [1917] considers that at present the total cholesterol content of the blood is practically of no diagnostic significance.

Though much work has been done on the total cholesterol content of blood or serum, comparatively little attention has been directed to the relative amounts of so-called free or non-esterified cholesterol and cholesterol in ester form. This unfortunate position is to some extent explained by the historical development of our knowledge of this subject. Fat, cholesterol and allied substances were obtained together by the extraction of tissues and fluids of the body by means of ether and other solvents, and in early days no effective methods were available for differentiating, quantitatively at any rate, between free and ester cholesterol. Consequently cholesterol came to be regarded as intimately connected with fat metabolism.

This idea has persisted throughout the history of the subject and has been upheld largely by the French observers. Terroine [1919] considers that there is a constant lipaemic coefficient, *i.e.* a constant ratio of total cholesterol to total fatty acid in individual animals. Chauffard and his school [1920]

consider that "normally there exists a tendency in the organism to preserve the equilibrium between the different elements of the lipaemia, fats, cholesterol and lecithin, and that it is probable that there exists a regulator mechanism which controls at the same time the amounts of the fats and lipins and the relative proportion of these different elements amongst themselves."

Further, Chauffard and his collaborators consider that cholesterol metabolism depends on three factors: (1) alimentation, (2) the glands of internal secretion, particularly the suprarenal cortex, (3) the liver. However, this view does not appear to lay sufficient stress on the fact that sterols are integral constituents of all living cells.

That so much attention has been concentrated on the total cholesterol of blood would seem to imply that the existence of a portion of this total in the form of ester is a purely fortuitous condition of no particular importance. However, a consideration of the different distribution of free and ester cholesterol throughout the body, noticeably in the brain, red blood corpuscles and in the bile would suggest that such an assumption is untenable.

In recent years cholesterol balance experiments by Gardner and Fox [1921, 1, 2; 1925] and by Thannhauser [1923] have shown that in the normal human adult there is always a small negative balance, that is, an excess of output over intake, which indicates that though the food may be a source of cholesterol, nevertheless there must be some synthesis in the body. That such synthesis does take place has been shown by the study of the relative growth and cholesterol content of infants' brain and by experiments with dogs by Beumer and Lehmann [1923], and with rats by Channon [1925] and by Randles and Knudson [1925].

Beumer [1923] goes so far as to advance the view that cholesterol is not to be considered as a food in any sense of the term but that in metabolism it plays a part more comparable with the rôle of the purines, in the sense that the cholesterol excreted by the organism is the true residuum, unrequired by the body, of the metabolic processes and is derived like uric acid from endogenous and exogenous sources. Whatever may be the truth of this conception it does not appear to us to modify the views previously maintained by Gardner in former papers of the nature of the cholesterol cycle.

In considering these different views it does appear to be certain that at the present time we have not the data on which to base a sound theory, nor data enough to criticise these views.

It appeared to us therefore to be of great immediate importance to study the free and ester cholesterol content of the plasma of normal individuals, and to examine the limits of its physiological variations and the conditions that cause such variations, also, if possible, to get evidence of the actual state of existence of the cholesterol in the blood. A general survey of the results hitherto obtained seems to justify the view that changes in the total cholesterol content of blood, or of the ratio of the free to ester forms, take place mainly in the plasma, and that the content of corpuscles is relatively if not

quite constant, and consists mainly of free cholesterol. In this paper we give the results of a number of analyses of the plasma of normal subjects.

#### TECHNIQUE.

The methods in use for estimating cholesterol and cholesterol esters in various tissues were critically dealt with by Gardner and Williams [1921] and by Gardner and Fox [1921, 1, 1924], and our experience has not led to the modification of the conclusions expressed in these papers. As the analyses of blood and plasma present rather more difficulties than those of other tissues, when both free and ester cholesterol are to be estimated, and rather conflicting statements have been made in recent years, notably by Bloor and Knudson [1916, 1917] and by Richter-Quittner [1920], we think it may be useful to give a more detailed description of our methods than has hitherto been published.

In our experience two methods of extraction are practically equally effective, viz., the method of Fex [1920] and the alcohol-ether method advocated by Bloor. In the case of whole blood about 10 cc. of blood are withdrawn from a vein and treated at once. If the plasma is to be analysed, it is better to draw a sufficient quantity of blood to yield at least 10 cc. of plasma into a tube containing a small quantity of solid potassium oxalate (0.2 %).

The blood is at once centrifuged and the plasma separated and weighed. A convenient quantity is about 10 to 15 g. It has been objected by Richter-Quittner that sodium oxalate and sodium fluoride tend to injure the corpuscles and should not be used. He recommends sodium citrate. In our experience, however, if the blood is centrifuged soon after drawing, the plasma shows no trace of haemolysis.

In Fex's procedure the fluid—whole blood or plasma—is diluted with about 10 to 15 times its volume of 2 % caustic soda, and after standing for several hours is heated for  $\frac{1}{2}$  to 1 hour on the water-bath. After cooling the fluid is thoroughly extracted in a separator by ether. This is best done as recommended by Fex; the alkaline fluid is placed in the separator with about half its bulk of ether, and thoroughly shaken at intervals during the day. After standing over night the ethereal solution is separated, replaced by a new quantity of ether and the alkaline fluid and the ether again shaken at intervals during the day. This process is repeated four or more times, until the ether no longer takes up anything from the alkaline fluid. The clear ethereal solution is then distilled to dryness on the water-bath, and the residue taken up in alcohol.

In the other process the weighed blood or plasma is run in a thin stream into about 250 cc. of a mixture of three parts alcohol to one part ether, which is kept gently rotating. The fluid is then heated to boiling on the water-bath. After cooling, the liquid is filtered through a small filter and the proteins very thoroughly washed by decantation with at first the alcohol-ether mixture and then with ether, any protein on the filter being returned at each washing to the flask: the protein is then washed with boiling alcohol and finally again

with ether. The ether-alcohol mixture is then distilled to dryness on the water-bath. The dry residue is boiled out thoroughly with small successive quantities of alcohol and the alcoholic extracts passed through a small filter. In this case it is necessary to filter, as the original ether-alcohol solution contains traces of protein and some of the plasma salts, which are not dissolved by the alcohol. At this stage it is advisable to wash the residue on the filter with ether and put the ether extract into the flask containing the residue insoluble in alcohol. The subsequent hydrolysis of the esters of cholesterol may then be done in this flask. Esters of cholesterol are not very soluble in alcohol, and this procedure obviates any loss of ester.

The free and ester cholesterol in the alcoholic solution, prepared by either method, are estimated by means of digitonin. Our procedure is as follows. The hot alcoholic solution is mixed with an excess of an alcoholic solution of digitonin in a small beaker and brought to boiling point. After cooling, water is added drop by drop until the alcohol is sufficiently diluted for the cholesterol digitonide to begin to deposit. After standing over night the fluid is carefully evaporated to dryness on the top of a water-bath. The contents of the beaker are then treated with three successive portions of 15 to 20 cc. of dry ether and these ether washings filtered through a weighed Gooch crucible and carefully collected. In this way the cholesterol esters along with any resinous matter are obtained in ethereal solution. The digitonide of the free cholesterol is then treated with boiling water to dissolve excess of digitonin, and brought on to the Gooch crucible and the washing completed with hot water. The filtrate should be water-clear, otherwise refiltered until it is. This washing process is complete when the washings, on vigorous shaking, show no trace of frothing. The Gooch crucible and its contents are then dried first at 100° and finally at 110° to constant weight.

The ethereal solution of the esters, etc., is poured into a flask provided with a reflux condenser (for this purpose the flask which held the original ether or ether-alcohol extract is used) and a large excess of an alcoholic solution of sodium ethoxide added, and after standing over night is boiled for at least 8 hours. After cooling, the alcoholic solution is largely diluted with ether and shaken in a separator with water. The aqueous solution so obtained is extracted three times with ether and the ether layer added to the main ether solution. The alkaline fluid is then again extracted in a second separator with a comparatively large quantity of ether, and the alkaline fluid then run off. The main ethereal solution formed by hydrolysis of the esters is then repeatedly shaken with successive portions of water to remove any traces of soap, and the aqueous washings shaken with the ether left in the second separator to avoid any risk of loss of cholesterol. The whole of the ether solution is then distilled to dryness on the water-bath, the residue taken up in alcohol and the cholesterol precipitated with digitonin and treated as described above.

This procedure presents advantages over the original method of Windaus [1910] which is used by most observers, especially in the case of fluids such

as blood which contain only small quantities of cholesterol or cholesterol esters, since any loss of cholesterol digitonide through slight solubility in alcohol is obviated. Such loss in the Windaus process is difficult to allow for, particularly when the quantity of cholesterol digitonide is small. The error due to this solubility is obviously the more serious the smaller the quantity to be weighed.

### RESULTS.

In Table I we give the results of a few duplicate analyses, both by Fex's and the alcohol-ether methods, which give an idea of the experimental errors to which the method of analysis is liable when quantities of the order mentioned are dealt with. When the complexity of the operation involved in the analyses is taken into account, we consider these results satisfactory.

Table I.

Method of extraction	Wt. of whole blood or plasma in g.	Wt. of cholesterol digitonide (g.) from		Cholesterol (g.) per 100 g. of whole blood or plasma			Remarks
		(a) Free cholesterol	(b) Ester cholesterol	Free	Ester	Total	
Fex ...	{ 10-3846	0-0378	0-0244	0-0885	0-0571	0-1456	Whole blood from normal male
	{ 10-3802	0-0384	0-0244	0-0899	0-0576	0-1475	
Alcohol-ether	8-3461	0-0402	0-0246	0-1171	0-0716	0-1887	Whole blood taken from a case of mitral stenosis
Fex ...	{ 10-4383	0-0462	0-0320	0-1076	0-0745	0-1821	
	{ 10-3287	0-0453	0-0308	0-1069	0-0727	0-1796	
Alcohol-ether	{ 15-531	0-0414	0-0706	0-0648	0-1105	0-1753	Plasma from a case of myocarditis
	{ 15-531	0-0423	0-0770	0-0662	0-1205	0-1867	
Fex ...	{ 15-531	0-0402	0-0742	0-0629	0-1161	0-1790	
	{ 15-531	0-0389	0-0705	0-0609	0-1104	0-1713	

Though these two methods of extraction give results in practical agreement, and in our experience are the best available when both free and ester cholesterol have to be estimated, nevertheless they are not perfect. Small quantities of cholesterol or cholesterol esters remain persistently in the proteins as the following experiments, selected from many, show.

#### *Alcohol-ether method.*

1. The protein collected after extraction from six cases, representing 72.7 g. of plasma, was dissolved in 2 % caustic soda and repeatedly extracted with ether in the manner described above. The ether extract gave 0.0009 g. of compound from free cholesterol, and 0.0011 g. from ester cholesterol. The extracted alkaline protein was then evaporated to dryness and boiled for 4 to 5 hours with glacial acetic acid containing hydrochloric acid. The acid liquor was then neutralised and thoroughly extracted with ether. The ether extract was then hydrolysed by sodium ethoxide to saponify any cholesterol acetate, and again extracted. The ether extract yielded 0.0026 g. of cholesterol digitonide. These figures correspond to 0.00031 % of free cholesterol, and 0.00038 % of ester cholesterol left in the protein and 0.0009 % freed by acid hydrolysis.

2. 7.95 g. of dried protein from 114.3 g. of extracted plasma of nine cases were treated in a similar manner. 0.0045 g. of compound from free cholesterol, 0.0061 g. from ester cholesterol and a trace after acid hydrolysis were obtained. These figures represent 0.00096 % free cholesterol and 0.0013 % ester cholesterol left in the original plasma.

*Fex method.*

3. 50.15 g. extracted plasma from five cases were very thoroughly re-extracted with ether, and the ethereal extract hydrolysed by sodium ethoxide and again extracted. From this 0.0058 g. of compound was obtained, equivalent to 0.0028 % of cholesterol (free and combined). After acid hydrolysis, as above, an extract was obtained which yielded 0.0012 g. compound equivalent to 0.0006 % cholesterol. The average error in these cases due to inefficient extraction was therefore 0.0034 g. per 100 g.

4. 93.1 g. of extracted plasma from seven cases were very thoroughly re-extracted with ether and the extract hydrolysed. From the extract was obtained 0.0011 g. compound, equivalent to 0.00029 g. cholesterol (free and ester) per 100 g. of original plasma. After acid hydrolysis it yielded 0.0054 g. compound, equivalent to 0.0014 g. cholesterol per 100 g. plasma.

These average errors due to inefficient extraction are obviously negligible, unless perchance they happened to be all concentrated in one sample, which, owing to the care taken, was very improbable, and are probably well within the variations caused by changes in the plasma concentration during the day.

*Cholesterol content of plasma of normal individuals.*

The subjects of our experiments were drawn chiefly from students in our classes at the London School of Medicine for Women and at St George's Hospital, from nurses, laboratory attendants and colleagues. They were all in normal health, as far as could be judged clinically. The samples of blood were drawn from an arm vein, and were collected in a few cases from fasting patients, *i.e.* before the first meal of the day, but in most cases about 4 to 5 hours after breakfast or a light lunch. In Table II we give essential details and results of our experiments.

DISCUSSION OF RESULTS.

Analyses of the plasma of normal human subjects have been published in recent years by Bloor and Knudson and by Richter-Quittner, who came to rather opposite conclusions. Bloor and Knudson [1917] examined 17 men and 9 women. The total cholesterol in plasma was determined by the colorimetric method of Bloor, and the ester cholesterol by the same method after elimination of free cholesterol by means of digitonin. The colorimetric method of Bloor [1916] is well known to give higher results than the digitonin method, and their average results are, as might be expected, higher than our own. They conclude that in normal human plasma there is a constant relation

Table II.

*Women*

No. of case	Age of subject	Time after last meal Hours	Nature of meal	Catamenia Between periods	Anticoagulant used K oxalate	Appearance of plasma Clear, no haemolysis Yellow, sl. opalescence	Method of extraction Fex Alcohol-ether	Wt. of plasma in g.	Wt. of digitonide (g.) from		Amount of cholesterol (g.) in 100 g. of plasma			Ratio free : ester
1	20	4	Breakfast	"	"	Clear, no haemolysis	"	11.1515	Free cholesterol	Ester cholesterol	Free	Ester	Total	Ratio free : ester
2	19	4	"	"	"	"	"	10.1138	Lost	0.0457	—	0.0896	—	1 : 3.97
3	19	4	"	"	"	"	"	13.2724	0.0137	0.0544	0.0323	0.1308	0.1637	1 : 2.8
4	21	5	"	"	"	"	"	13.1465	0.0112	0.0314	0.0205	0.0575	0.0780	1 : 3.82
5	20	3	"	"	"	"	"	18.4537	0.0223	0.0738	0.0412	0.1365	0.1777	1 : 3.32
6	20	4	"	"	"	Yellow, sl. opalescence	"	14.2678	0.0323	0.0489	0.0426	0.0644	0.1070	1 : 1.52
7	19	5½	"	"	"	"	"	12.6801	0.0210	0.0319	0.0358	0.0541	0.0802	1 : 1.42
8	18	4	"	"	"	"	"	7.4765	0.0234	0.0509	0.0563	0.0875	0.1538	1 : 1.73
9	25	4	"	"	"	Clear, v. sl. haemolysis	"	9.4117	0.0231	0.0283	0.0597	0.0820	0.1517	1 : 1.54
10	19	4½	"	"	"	Opalesc. sl. haemolysis	"	8.464	0.0215	0.0206	0.0328	0.0597	0.1215	1 : 1.85
11	24	4½	"	"	"	Clear, sl. haemolysis	"	9.5207	0.0232	0.0485	0.0599	0.1108	0.1707	1 : 1.61
12	17	4½	"	"	"	Opalesc. sl. haemolysis	"	6.6325	0.0181	0.0282	0.0663	0.1070	0.1733	1 : 2.08
13	24	4	"	"	"	Clear, v. sl. haemolysis	"	10.9658	0.0304	0.0631	0.0675	0.1402	0.2077	1 : 2.08
14	23	1½	Light breakfast Light lunch	"	Paraffined tube K oxalate	Clear, no haemolysis	"	10.5254	0.0328	0.0393	0.0758	0.0808	0.1566	1 : 1.20
15	22	Imme- diately	Good lunch Lunch	"	"	Yellow, clear	"	7.5215	0.0172	0.0313	0.0556	0.1012	0.1568	1 : 1.82
16	20	4½	Light breakfast	Period now on	"	Brownish, clear, no haemolysis	"	12.3943	0.0288	0.0538	0.0565	0.1055	0.1620	1 : 1.90
17	19	6	Breakfast	Sl. amenorrhoea	"	Clear, v. sl. haemolysis	"	13.3648	0.0519	0.0727	0.0944	0.1322	0.2266	1 : 1.40
18	18	5½	"	Between periods	"	Sl. opalesc., no haemol.	"	12.1687	0.0356	0.0500	0.0713	0.1001	0.1714	1 : 1.40
19	20	5½	"	Sl. amenorrhoea	"	Clear, no haemolysis	"	14.2066	0.0252	0.0477	0.0431	0.0816	0.1241	1 : 1.69
20	19	4½	"	Between periods	"	Clear, sl. haemolysis	"	12.1956	0.0243	0.0690	0.0458	0.1302	0.1760	1 : 2.84
21	18	4½	Light breakfast	"	"	"	"	7.4523	0.0106	0.0261	0.0346	0.0617	0.1263	1 : 2.65
									Mean		0.0541	0.0986	0.1528	

## Men

[illegible]

<sup>1</sup> The extracted alkaline plasma from these 7 cases, on re-extraction and subsequent hydrolysis, gave 0.0011 g. compound equivalent to 0.00029 g. % plasma. After acid hydrolysis gave 0.0044 g. compound equivalent to 0.0014 g. % plasma.

1. The extracted alkaline plasma from these 7 cases, on re-extraction and subsequent re-extraction, yielded 0.0034 g. compound equivalent to 0.0014 g. % plasma.

\* Precipitated protein from these 6 cases dissolved in 2 % NaOH and thoroughly extracted with ether gave 0.0009 g. compound from free cholesterol and 0.0011 g. compound from ester cholesterol equivalent to 0.0026 g. free cholesterol per 100 g. plasma. After acid hydrolysis gave 0.0026 g. compound equivalent to 0.0009 g. free cholesterol per 100 g. plasma. After acid hydrolysis gave 0.0038 g. ester cholesterol per 100 g. plasma. After acid hydrolysis gave 0.0028 g. compound equivalent to 0.0028 g. per 100 g. plasma.

<sup>c</sup> Total alkaline plasma of these 5 cases, after re-extraction and subsequent hydrolysis, gave 0.0028 g. compound equivalent to 0.0028 g. per 100 g. plasma.

\* Total serum plasma volume was 600 ml.  
• 0.0012 g. compound equivalent to 0.00058 g. per 100 g. plasma.  
• Mixed sample from 12 subjects.  
• Mixed sample of about equal volumes from 6 subjects.  
• After acid hydrolysis 0.0924 g. compound equivalent to 0.0058 %.

- a Mixed sample from 12 subjects.
- b Mixed sample of about equal volumes from 6 subjects.
- c Mixed sample of about equal volumes from 8 subjects.
- d Mixed sample of about equal volumes from 0.0028 g. compound found equivalent to 0.0028 g. free cholesterol but 0.0028 g. compound from ester cholesterol found equivalent to 0.0048 % plasma.

- <sup>7</sup> On re-extraction, no free cholesterol but 0.0028 g. compound from ester cholesterol found equivalent to 0.0067 %.
- <sup>8</sup> The protein dissolved in 2 % NaOH and thoroughly extracted. No free cholesterol but 0.0018 g. compound from ester cholesterol equivalent to 0.0048 % plasma.
- <sup>9</sup> Mixed sample of about equal volumes from 0.0025 g. ester.

- On re-injection, no rise in blood cholesterol was observed.
- The protein dissolved in 2 % NaOH and thoroughly extracted.
- No free cholesterol but 0.0018 g. compound from each cholesterol equivalent to 0.00017 g. 1.
- Amino somewhat cyanosed on venipuncture.

- Am somewhat cyanosed on venipuncture.



between free cholesterol and ester cholesterol, the ester cholesterol being 58 % of the total, with a variation from the average in individuals of about 15 % of the average; and further that there is no notable difference in their values between men and women. This constant relation between free and bound cholesterol they consider gives further support to the assumption that cholesterol takes an active part in fat metabolism, and also indicates that for cholesterol esters, as for other "lipoids," there is an efficient regulation. We scarcely think these general conclusions are warranted by their figures, still less by our own. Bloor and Knudson find that the ratio of free to ester cholesterol is on an average 1 : 1.35 in normal men, with individual limits of variation of 1 : 0.84 to 1 : 2.14, and in normal women an average of 1 : 1.49 with limits of 1 : 1.04 to 1 : 2.34. As will be seen from Table II our own average ratios are for men 1 : 2.36, with limits of 1 : 0.82 to 1 : 21.3, or eliminating three cases in which the cholesterol was nearly all in the form of ester, 1 : 2.36 with limits of 1 : 0.82 to 1 : 3.29; and in women an average of 1 : 1.93 with variations of 1 : 0.97 to 1 : 3.97.

On the other hand, Richter-Quittner [1920] on, we think, insufficient normal data, concluded that blood corpuscles contain exclusively free cholesterol and no ester, whereas the *plasma contains exclusively ester and no free cholesterol*. He also draws a distinction between alimentary hypercholesterolaemia and pathological hypercholesterolaemia, but this subject we hope to deal with in another communication.

Richter-Quittner appears to attribute the varying results of other observers to injury to corpuscles in obtaining the plasma, by such causes as too vigorous centrifugalisation, and, particularly, to the use of sodium oxalate or fluoride as anticoagulants, since these salts even in small amounts act as poisons and harm the red cells. In our experiments we were careful to reduce disturbance by centrifugalisation to a minimum. In most experiments, however, we used oxalates in the minimum quantity to prevent coagulation during the short interval between drawing the blood and preparing the plasma. In a great majority of the cases the plasma obtained was yellow and untinged with red, so that, as far as absence of haemolysis is an indication, the red cells were unharmed. However, to test this criticism, we drew the blood in cases 13, 34, 35 into a paraffin lined tube and centrifuged at once without any anticoagulant, in cases 37 and 40 we used heparin (a substitute for hirudin) and in cases 41, 42 and 43 potassium citrate, as recommended by Richter-Quittner. The results, as will be seen from Table II, in no wise differ from those obtained with oxalate.

It is clear from our results that normal individuals differ widely both in the total cholesterol of their plasma, and in the ratio of so-called free cholesterol to cholesterol in the form of esters.

These variations could scarcely be due to the direct absorption of cholesterol from the food into the blood plasma, since, with a few exceptions, the results were obtained from blood after a fast, or several hours after a light

meal, such as breakfast, containing but a small absolute amount of cholesterol; for even if we assume that the food contained 0.15 g. of cholesterol—which is quite an outside figure—and if we assume that the whole of this found its way into the blood stream, this would, in an individual with, say, 6 litres of blood, only increase the cholesterol by 0.00025 g. %, a figure well within the error of experiment.

*Red blood corpuscles.*

Richter-Quittner is probably right in his contention that the red cells contain mainly free cholesterol, and in this he is in agreement with Bloor and Knudson and other observers. The method usually adopted is to calculate the composition of the red cells from analyses of the whole blood and plasma, together with a determination of the ratio of corpuscles to plasma by the haematocrit or other method. The accumulation of experimental errors involved in such a computation is however too great to indicate, with certainty, the presence of small quantities of ester. A more satisfactory method of proving the presence of ester is a direct analysis of the *thoroughly* washed corpuscles. The cholesterol is more easily extracted from corpuscles than from plasma, and, in this respect, they resemble other tissues. The first thorough and reliable investigation of animal blood was made by Wacker and Hueck [1913] who found that in horse blood the dried red cells contained 0.36 % free and no ester cholesterol, whilst the dried white cells contained 1.729 % free and 0.04 % ester. Beumer and Bürger [1913] also found that human blood cells contain only free cholesterol, though in certain diseases traces of ester appear, but in such small amount that the authors rightly consider the values should be accepted with caution.

We made only one experiment—in the case of mixed blood No. 31—and found after elaborate washing of the corpuscles 0.0036, 0.0018 and 0.0012 % of ester cholesterol in the moist corpuscles: but as the quantities of cholesterol-digitonide weighed were only 2.0, 0.8 and 1.2 mg. respectively we cannot place much reliance on the figures, except that they indicate a small positive precipitate.

An objection might be raised against the direct method that, during the somewhat lengthy process of washing, autolytic changes may have resulted in a loss or gain of ester cholesterol, but this scarcely seems likely as calculation from whole blood and plasma points to the same results. Furthermore, a large mass of thoroughly washed corpuscles which had been kept 4 months in a stoppered bottle, and had undergone considerable autolytic change, was analysed with the following results: 13.8 g. moist material gave 45.4 mg. digitonin-compound from free cholesterol, and 2.2 mg. from ester cholesterol, the latter equivalent to 0.0039 %. Evidently the cholesterol had not been appreciably changed during autolysis. The bulk of the material (73 g.) was extracted, and the cholesterol isolated and converted into benzoate. 0.356 g. of pure benzoate, melting to a turbid liquid at 145°–146° and clearing at 178°, was obtained.

We take this opportunity to thank the Royal Society Grant Committee for help in the purchase of digitonin, and our pupils and colleagues for samples of blood.

## REFERENCES.

- Beumer (1923). *Z. ges. exp. Med.* **35**, 328.  
Beumer and Bürger (1913). *Arch. exp. Path. Pharm.* **71**, 316.  
Beumer and Lehmann (1923). *Z. ges. exp. Med.* **37**, 274.  
Bloor (1916). *J. Biol. Chem.* **24**, 227.  
Bloor and Knudson (1916). *J. Biol. Chem.* **27**, 107.  
—— — (1917). *J. Biol. Chem.* **29**, 7.  
Channon (1925). *Biochem. J.* **19**, 424.  
Chauffard, Laroche and Grigaut (1920). *Ann. Med.* **8**, 69, 149, 321.  
Denis (1917). *J. Biol. Chem.* **29**, 93.  
Fex (1920). *Biochem. Z.* **104**, 82.  
Gardner and Fox (1921, 1). *Biochem. J.* **15**, 376.  
—— — (1921, 2). *Proc. Roy. Soc. Lond. B*, **92**, 358.  
—— — (1924). *Biochem. J.* **18**, 1058.  
—— — (1925). *Proc. Roy. Soc. Lond. B*, **98**, 76.  
Gardner and Williams (1921). *Biochem. J.* **15**, 363.  
Randles and Knudson (1925). *J. Biol. Chem.* **66**, 459.  
Richter-Quittner (1920). *Wien. Arch. inn. Med.* **1**, 425.  
Terroine (1919). Contribution à la Connaissance de la Physiologie des Substances grasses et lipoldiques. Masson et Cie., Paris, p. 334.  
Thannhauser (1923). *Z. physiol. Chem.* **127**, 278.  
Wacker and Hueck (1913). *Arch. exp. Path. Pharm.* **74**, 416.  
Windaus (1910). *Z. physiol. Chem.* **65**, 110.

# XXI. STUDIES ON THE CHOLESTEROL CONTENT OF NORMAL HUMAN PLASMA. PART II.

## THE ATTRACTION OF THE PROTEINS OF PLASMA FOR STEROLS.

By JOHN ADDYMAN GARDNER AND HUGH GAINSBOROUGH.

*From the Biochemical Department, St George's Hospital, London.*

*(Report to the Medical Research Council.)*

*(Received December 23rd, 1926.)*

It has long been recognised that the whole of the cholesterol and cholesterol esters cannot be separated from blood plasma and serum by simple shaking with ether.

That, in native serum, the ether-soluble constituents are associated in some way with the serum proteins would appear to follow from the work of Hardy [1905], Haslam [1913] and Chick [1914], and, as they are present in relatively very small amounts they acquire the solubility properties of proteins. As Hartley [1915] has pointed out, when the conditions are reversed, traces of proteins may acquire the solubility properties of the "lipoid" with which they are associated.

As far as we are aware, Bang was the first to suggest that the cholesterol of serum may be in combination with proteins, and most probably with the globulins. A verbal communication by Bang on the subject is quoted by Thaysen [1913], and Bang [1918] gave two analyses of dog's globulin and one of human in which considerable proportions of cholesterol were found, and suggested that this might form a useful subject for further study.

In a series of papers published by Handovsky, Lohmann and Bosse [1925], Bosse [1925], Bosse and Handovsky [1925], Handovsky [1925], Handovsky and Lohmann [1925], these authors determined the total cholesterol in a large number of ox-sera, and found also that on precipitating the globulins by various salts a proportion of the whole sterol was precipitated along with the globulins. They concluded that the globulin has a greater attraction for cholesterol in serum than the albumin, and attempted to gain an idea of the quantity of cholesterol in association with the globulin, and the firmness of the union, by determining the proportion of the whole sterol which can be obtained by directly shaking out the serum with ether under rigid but arbitrary conditions.

Their figures show that when the albumin fraction of the protein is high the amount that can be directly shaken out with ether is high, but when the albumin fraction is low, relatively to the globulin, the percentage of the whole cholesterol that can be shaken out with ether is low. They concluded that in normal ox-sera about 25 % of the total cholesterol is firmly bound to globulin; the rest should be bound to phosphatides. The intensity of this union, measured by the capacity of being directly shaken out with ether, is dependent on the salt and water content of the sera. They took however no account of the fact that cholesterol is present both in the free and ester conditions. Young [1922] on the basis of his polariscopic investigation assumed that the cholesterol esters are associated in a loose way with the serum-albumin. Cholesterol is precipitated with these and can, both from the serum-albumin and also from the serum, be extracted with light petroleum or with ether. Troensegaard and Koudall [1926] obtained some evidence of the existence of cholesterol as a prosthetic group in serum-globulin, from which by simple extraction no cholesterol was obtained, by the fractionation of the acetyl bases of the different blood proteins. From serum-globulin they obtained hydrocarbons  $C_{16}H_{28}$  and  $C_{18}H_{32}$  which they considered must have originated from a cholesterol complex present in the globulin, since they were not obtained from either globin or albumin.

Much more work will evidently have to be done on this reaction before their conclusions can be regarded as firmly established.

In the same year Theorell [1926] analysed the proteins precipitated from horse plasma by fractional saturation with ammonium sulphate. These fractions, even after dialysis and redissolution, were found to contain considerable quantities of lipins. 77 % of the total cholesterol of the plasma was thus precipitated.

The ratios of the cholesterol to ether-soluble phosphorus of fibrinogen, globulin and albumin fractions were respectively 20.5, 16.9 and 9.7, which may be taken as an indication of the distribution of cholesterol and phosphatides between these fractions. In this connection we may point out that Wacker and Hueck [1913] in very careful experiments with horse blood showed that pure fibrin contains no cholesterol, though commercial samples do contain small amounts.

For some time past we have been working on this problem, and in view of the recent papers quoted above, we think it may be of interest to give an account of our work to date.

Cholesterol is quite insoluble in water, in alkalis and in dilute mineral acids, but Porges and Neubauer [1908] found that colloidal suspensions in water may be prepared by adding a solution of cholesterol in acetone to water and dialysing. They concluded from the properties of the fluid that it was an anodic suspensoid. This work was verified by Partington [1911] who observed that a solution of cholesterol in alcohol gave a colloidal solution when added in small quantities to water with stirring. The alcohol was removed by

dialysis and the colloidal solution was left for several months without very marked deposition. It was immediately precipitated by mineral acids, fixed alkalis and by various salts. We find that similar colloidal suspensions, though more dilute, may be made by dropping an alcohol or an acetone solution into boiling physiological saline. Similar colloidal solutions of esters, such as cholesterol palmitate, may also be readily prepared. Such colloidal solutions are markedly opalescent, whereas plasmas, containing much larger concentrations of cholesterol, are often perfectly clear and transparent to the eye, so that in plasma even if the cholesterol be not in chemical or physical union with other constituents, it must be in a quite different state to that in the artificial suspensions.

Colloidal suspensions of cholesterol, even when very dilute, we find are readily precipitated by half saturation with ammonium sulphate, and the precipitation is complete. For example, 250 cc. of a colloidal suspension of cholesterol in saline, containing 0.0055 g. cholesterol, was half saturated with ammonium sulphate, the precipitate separated and dissolved in ether and estimated by means of digitonin. It gave 0.0246 g. cholesterol digitonide, equivalent to 0.00598 g. of cholesterol. Similar colloidal suspensions of cholesterol palmitate are also flocculated by half saturation with ammonium sulphate, but we are not sure that the precipitation is quite complete. For example, 200 cc. of a suspension of palmitate on evaporation to dryness and solution in alcohol yielded 0.0211 g. cholesterol digitonide, equivalent to 0.00256 %. 250 cc. of the same suspension after half saturation with ammonium sulphate yielded a precipitate which on solution in alcohol and precipitation with digitonin gave 0.0208 g. compound, equivalent to 0.00202 %. Evidently approximately 22 % of the cholesterol palmitate was not precipitated. The actual differences in weight however are not much beyond the limits of experimental error.

If the cholesterol in plasma is in a similar state of colloidal suspension we should expect the whole or almost the whole would be precipitated along with the globulin on half saturation of the plasma with ammonium sulphate.

In order to gain some idea of the proportion of the free cholesterol and cholesterol esters thrown down on simple separation of the proteins by means of ammonium sulphate, 102 g. of plasma from a case of hyperpiesia, which analysis had shown to contain 0.087 % of free cholesterol and 0.166 % in ester form, were diluted with an equal bulk of water, and precipitated by the addition of 204 cc. of saturated ammonium sulphate. The precipitated globulins were filtered and well washed with half saturated ammonium sulphate solution. The filtrate and washings were then saturated with ammonium sulphate, and the precipitated albumins filtered and washed with a saturated solution of ammonium sulphate. The globulin precipitate was then separated into euglobulin and pseudoglobulin by digestion with saturated sodium chloride solution. The proteins thus separated were not dried and weighed, but were at once dissolved in 2 % caustic soda solution and extracted with

ether according to the method of Fex [1920]. The free and ester cholesterol were then determined in the various ether extracts, with the following results.

Table I.

Protein	Wt. of digitonin comp. in g. from		Sterols as % of original plasma			Sterols as % of cholesterol of plasma		
	Free chole-sterol	Ester chole-sterol	Free chole-sterol	Ester chole-sterol	Total chole-sterol	Free chole-sterol	Ester chole-sterol	Total chole-sterol
Euglobulin	0.0826	0.0768	0.0197	0.0183	0.0380	22.6	11.0	15.0
Pseudoglobulin	0.0648	0.1136	0.0155	0.0271	0.0426	17.8	16.3	16.8
Albumin	0.1771	0.3258	0.0422	0.0777	0.1199	48.5	46.8	47.4
			0.0774	0.1231	0.2005	88.9	74.1	79.2

The protein-free filtrates were thoroughly extracted with ether, but no sterol, either free or ester, was obtained. On boiling these filtrates with acetic acid for many hours, and re-extraction, a minute portion of cholesterol was obtained, weighing in the form of digitonin compound only 1.4 mg.

From this experiment it is clear that the whole of the cholesterol and cholesterol esters is thrown down with the proteins on precipitation with ammonium sulphate. There is a loss however of about 20 % of the sterol, in the proportion of about 1 of free to 2.3 of ester. The loss must represent sterol so intimately associated with one or other of the proteins as to be difficult of extraction by shaking the alkaline solution with ether.

Further experiments with other samples of plasma showed that by repeated re-resolution and re-precipitation of the protein fractions, the protein could be largely separated from the sterol thrown down with it in the initial precipitation; and also that the difficultly extractable portions were associated rather with the globulins than the albumin.

We therefore decided to obtain a large sample of blood from a normal individual, and subject the plasma to a more thorough fractionation.

Mr C. S. Coleman, a vigorous young man aged 19, in perfect health, volunteered to give us 450 cc. of blood, and we take this opportunity of expressing to him our sincere thanks.

The plasma was light brown in colour, clear, and showed no trace of haemolysis. On analysis it was found to contain 0.0445 % free, 0.1099 % ester and 0.0057 % of cholesterol isolated only by acid hydrolysis. The total was thus 0.1602 %. 200 cc. of this plasma were diluted to 1000 cc. with water and mixed with 1000 cc. of a saturated solution of ammonium sulphate. The globulins were filtered and washed with half saturated ammonium sulphate. The filtrate and washings were then saturated with ammonium sulphate and the albumin filtered and washed with saturated ammonium sulphate. The globulins were then dissolved in physiological saline and the albumin in water, carefully filtered, and made up to 1 litre each. The globulin solution was slightly opalescent and the albumin perfectly clear and transparent. The globulin solution was then again precipitated by half saturation with ammonium sulphate and the globulin washed. The filtrate and washings were

then saturated with ammonium sulphate and the precipitated albumin, after washing, dissolved in water and added to the main albumin solution. The aqueous albumin solution was then half saturated with ammonium sulphate and a small quantity of globulin which separated was added after washing to the main globulin fraction.

The albumin solution was then saturated with ammonium sulphate and the precipitated albumin filtered and washed with saturated ammonium sulphate. This process was then repeated a third time, when the albumin fraction separated from the globulin, and the globulin separated from the albumin fraction, were practically negligible.

The globulins were then re-dissolved in saline, made up to a litre and re-precipitated by half saturation with ammonium sulphate. This process was repeated four times. The albumin was then dissolved in a litre of water, carefully filtered and re-precipitated by saturation with ammonium sulphate. This process was also repeated four times. The final albumin dissolved in water, giving a perfectly clear and transparent solution.

The final globulin precipitate was then digested over night in a considerable bulk of saturated sodium chloride, and thus approximately separated into pseudoglobulin and euglobulin. This process was not repeated more than once.

The free protein fractions were then coagulated by heat in the usual way, thoroughly washed free from salts, dried *in vacuo* over sulphuric acid and weighed.

In this way 0.7129 g. of pseudoglobulin, 2.7077 g. of euglobulin and 10.133 g. of albumin were obtained.

These proteins were allowed to soak in 2 % caustic soda solution until they swelled to jelly, and were then heated on the water-bath with a suitable further quantity of the alkali, until they went into solution, which took half to one hour. After cooling, the alkaline fluids were thoroughly extracted with ether in the manner described for plasma in Part I, and the free and ester cholesterol determined by digitonin. The extracted alkaline fluids were then evaporated to dryness and heated for 5 to 6 hours with glacial acetic acid containing hydrochloric acid. The fluid was then diluted, neutralised and extracted again with ether. After distilling off the ether, the residue was hydrolysed by means of alcoholic sodium ethoxide to saponify any cholesterol acetate. After extraction the cholesterol set free by acid hydrolysis was precipitated by digitonin.

The results are summarised in Table II.

It was noticed that the surface of the filter papers used in filtering the globulin became covered with a varnish-like substance, insoluble in physiological salt solution. The papers were therefore cut up, thoroughly washed with saline, dried and extracted in an all-glass extraction apparatus with ether. On evaporating the ethereal solution, 0.495 g. of a brownish oil was obtained. This on treatment with digitonin yielded 0.2236 g. of compound



Table II.

Protein	Wt. of protein	Cholesterol digitonide in g. from			Cholesterol (g.) per 100 g. of protein				Cholesterol (g.) per 100 g. of original plasma			
		Free cholesterol*	Ester cholesterol	Cholesterol obtained by acid hydro- lysis	Free	Ester	By acid hydro- lysis	Total	Free	Ester	By acid hydro- lysis	Total
Euglobulin	2.7077	0.1064	0.0709	0.0050	0.9553	0.6365	0.0449	1.6367	0.0129	0.0086	0.0006	0.0221
Pseudoglobulin	0.7129	0.0031	0.0052	0.0053	0.1057	0.1773	0.1807	0.4637	0.00377	0.000632	0.000644	0.00105
Albumin	10.133	0.0082	0.0102	0.0016	0.0197	0.0245	0.0038	0.0480	0.0010	0.0012	0.0002	0.0024
									0.0143	0.0104	0.0014	0.02615
									By analysis of plasma			
									0.0445	0.1099	0.0057	0.1601

from free cholesterol, and 0.3872 g. from ester cholesterol. The oil, therefore, contained 10.98 % of free cholesterol and 19.02 % of ester cholesterol. The albumin filter papers were also cut up and, after being thoroughly washed with water, were dried and extracted with ether. 0.41 g. of a dark brown solid grease was obtained, from which 0.0797 g. digitonin compound of the free cholesterol and 0.1417 g. from the ester cholesterol were obtained. It therefore contained 4.72 % of free cholesterol and 8.4 % of ester cholesterol.

Expressed in g. per 100 g. of the original plasma, the free cholesterol on the globulin papers was 0.0272 and the ester 0.0471, and that on the albumin paper 0.0097 and 0.0172.

## DISCUSSION OF RESULTS.

**Summarising the results of the above experiment:**

Total sterol in 100 g. plasma		= 0.1601 g.
Found in proteins	0.0262 g.	} = 0.1274 g.
Found in filter paper	0.1012 g.	
		<u>Loss 0.0327 g.</u>

The loss of sterol in this experiment is no doubt in the small amounts of intermediate fractions of proteins not worked up, and in the ammonium sulphate mother liquors, which, owing to their vast bulk, were not extracted with ether.

The percentage of total plasma sterol retained in proteins	= 16·36
" " " euglobulin	= 13·80
" " " pseudoglobulin	= 1·03
" " " albumin	= 1·50

Evidently the greater portion of the sterol retained is in the euglobulin, and in this our results confirm those of Handovsky.

The quantity of total cholesterol in combination with 1 g. of euglobulin is 0.0166 g.; Handovsky gives 0.0084 to 0.016 g. for ox-serum. Of the total free cholesterol retained in the proteins, 90.34 % is in the euglobulin, 2.64 % in the pseudoglobulin and 7.0 % in the albumin, whilst of the total ester cholesterol retained in the protein, 82.4 % is in the euglobulin, 6.05 % in the pseudoglobulin and 11.5 % in the albumin.

It will be noticed from these figures that the different proteins show much the same power of retention for cholesterol in the free as in the ester state. We do not know whether it would have been possible to eliminate all occluded sterol from the pseudoglobulin and the albumin by a more extended resolution and re-precipitation, but it is highly unlikely that this would have been the case with the euglobulin.

It is difficult to form a precise picture from our results, or those of other workers, as to the true relationship between the sterols and the proteins in natural plasma, but apparently there must be a close association with the euglobulin. It is not possible to conclude that the union is chemical in the ordinary sense, but probably there is (in such complex substances) really no sharp distinction between so-called physical adsorption and chemical union of associated substances.

It seems not improbable that changes in the globulin albumin ratio in plasma may have something to do with the variations in sterol content of plasma in disease, and even under normal physiological conditions. We hope however to deal with this in another communication.

## REFERENCES

- Bang (1918). *Biochem. Z.* **90**, 383.  
Bosse (1925). *Pflüger's Arch.* **210**, 56.  
Bosse and Handovsky (1925). *Pflüger's Arch.* **210**, 50.  
Chick (1914). *Biochem. J.* **8**, 404.  
Fex (1920). *Biochem. Z.* **104**, 82.  
Handovsky (1925). *Pflüger's Arch.* **210**, 35.  
Handovsky and Lohmann (1925). *Pflüger's Arch.* **210**, 59.  
Handovsky, Lohmann and Bosse (1925). *Pflüger's Arch.* **210**, 63.  
Hardy (1905). *J. Physiol.* **33**, 251.  
Hartley (1915). *Brit. J. Exp. Path.* **6**, 180.  
Haslam (1913). *Biochem. J.* **7**, 492.  
Partington (1911). *J. Chem. Soc.* **99**, 318.  
Porges and Neubauer (1908). *Biochem. Z.* **7**, 152.  
Thaysen (1913). *Biochem. Z.* **62**, 110.  
Theorell (1926). *Biochem. Z.* **175**, 297.  
Troensegaard and Koudall (1926). *Z. physiol. Chem.* **153**, 111; **157**, 62.  
Wacker and Hueck (1913). *Arch. exp. Path. Pharm.* **74**, 416.  
Young (1922). *Proc. Roy. Soc. Lond. B*, **93**, 15.

## XXII. THE EFFECTS OF CHEMICAL AND PHYSICAL CHANGES IN ENVIRONMENT ON RESTING BACTERIA.

BY JUDA HIRSCH QUASTEL  
AND WALTER REGINALD WOOLDRIDGE  
(*Frank Smart Student, Gonville and Caius College*).

*From the Biochemical Laboratory, Cambridge.*

(Received December 24th, 1926.)

PRIOR and subsequent to the publication [Quastel, 1926] of certain views concerning the mechanism of oxidations induced by enzymes or tissues, experiments of a systematic nature have been carried out by us with the object of either substantiating or refuting these views.

Certain facts have been brought to light, one or two of which are, at present, difficult to interpret, but which in the main confirm the view that a seat of the many activations brought about by bacteria (*B. coli*) is associated with the surface structure of the cell. This does not imply, of course, that intracellular surface activations cannot occur. The facts, too, throw some light on the question of the specificity of enzyme action, and it will be shown that our views are in no way contradictory to or irreconcilable with the facts concerning specificity. Rather do they show that specificity of behaviour is to be anticipated; that it should be the rule and not the exception with biological activations.

Our experimental method, briefly, consists in exposing bacteria, whose activations of a certain number of substrates have been ascertained by the methylene blue technique, to varying physical and chemical conditions for definite periods of time. Such changes in environment produce effects on the organism which can be followed quantitatively by measuring the changes in the activations brought about by the treated organism. In this way a quantitative estimate of the effect of a certain environment on an organism can be obtained. Hitherto such an effect has been studied mainly from the point of view of antiseptic action. We find, however, that although exposure to a certain environment may have been lethal in the sense that few or none of the treated cells are capable of reproduction in the usual nutrient media, they are still capable of accomplishing a number of activations. This number diminishes and the activating power usually becomes less powerful as the length of exposure increases until eventually all activating power disappears.

Whether this experimental method will enable us to perceive precisely how an antiseptic exerts its lethal effect still remains to be seen, but it is clear that the method will allow us to compare and contrast the effects of certain lethal materials in a manner much more extensive than has hitherto been possible.

We have confined ourselves exclusively to the study of one organism, *B. coli*, four different strains having been used in the course of the work.

A stock suspension of resting *B. coli* is prepared in the following way. In each of a series of Roux bottles of 1100 cc. capacity are placed 150 cc. tryptic broth prepared according to Cole and Onslow's method [1916], and the bottles sterilised by autoclaving. Each of the contents of 10 bottles is now inoculated with 1 cc. of a fresh (18 hr.) tryptic broth culture of *B. coli* and the bottles are incubated at 37° for 40 to 48 hours. After this period the contents of the bottles are centrifuged and the clear supernatant liquid is decanted from the deposit of the organism. This deposit is suspended in 0.85 % pure salt solution and the suspension again centrifuged. This is repeated so that finally the organism has been twice washed. It is now suspended in 200 cc. of 0.85 % salt solution and vigorously aerated for 3 to 4 hours. This serves to produce a homogeneous suspension and to remove any easily oxidisable material which may be present. The bacterial suspension is placed in a flask plugged with wool and stored at 0°. Investigations with resting bacteria should be carried out within a fortnight of their preparation. After this period a new preparation should be made.

Activations by the bacteria are determined by the methylene blue method described in detail in earlier communications [Quastel and Whetham, 1925, 1, 2; Quastel and Wooldridge, 1925]. The stock suspension of bacteria, which should always be kept at 0°, may be diluted to suit the worker's needs. We find it convenient, for this work, to dilute the suspension to such an extent that 1 cc. will not reduce *in vacuo* 7 cc. 1/35,000 methylene blue solution at  $p_H$  7.4 and 45° in one hour and will, in presence of *M*/140 sodium succinate, reduce the same quantity of methylene blue under the same conditions in approximately 10 minutes.

The actual treatment of the organism is described fully in the experimental sections which follow. After treatment the organism is well washed by centrifuging and made up to its initial concentration, previous to treatment, with normal saline solution. The activations of the treated organism are now determined in exactly the same way as for the normal organism.

### I. *The variation of reduction velocity of methylene blue with quantity of organism.*

The time of reduction of a constant amount of methylene blue by a given donator is roughly inversely proportional to the quantity of organism present. This is shown by the value  $qt$  (Table I) where  $q$  = quantity of organism present and  $t$  = reduction time. The value is a fair constant with such

donators as formic acid and lactic acid. With succinic acid and glucose the value is only constant for relatively high concentrations of organism; as the quantity of organism becomes smaller the value  $qt$  rises rapidly. This divergence from the linear relation between reduction velocity and concentration of organism, as the latter approaches small values, is due to the relatively slow reduction which allows for at least two inhibiting effects on the organism to become appreciable and so to lengthen the time of reduction. These inhibiting effects are:

- (1) the action of methylene blue on the organism (see Section VI);
- (2) the action of temperature (45°) on the organism (see Section II).

Both the presence of methylene blue and a relatively high temperature tend, if long sustained, to produce inactivations in the organism which are relatively more marked with certain donators than with others. It is this circumstance which accounts for the relatively greater deviations from linearity exhibited by some donators than by others.

Table I.

Each vacuum tube contained 2 cc. phosphate buffer  $p_H$  7.4, 1 cc. 1/5000 methylene blue solution,  $q$  cc. of the suspension of organism, and the donator, the volume being made up to 7 cc. with freshly boiled saline solution. Reductions were carried out *in vacuo* at 45°. *Coli* 1, *coli* 2, etc., were different suspensions and strains of *B. coli*; results with *coli* 1 can be compared strictly quantitatively with one another but only qualitatively with *coli* 2, etc. All substances under investigation were brought to  $p_H$  7.2 by sodium hydroxide or hydrochloric acid. Controls with all organisms (absence of donators) showed no reduction within 2 hours. The reduction time  $t$  is given in minutes. In this table, as elsewhere in this paper, the sign  $\infty$  indicates (except where otherwise stated) that reduction does not occur within 2 hours. The following are typical results.

M/140 succinic acid <i>Coli</i> 1			M/140 lactic acid <i>Coli</i> 1			M/140 formic acid <i>Coli</i> 2			M/140 glucose <i>Coli</i> 3		
$q$	$t$	$qt$	$q$	$t$	$qt$	$q$	$t$	$qt$	$q$	$t$	$qt$
1	25	25	0.5	27.8	13.9	0.1	33	3.3	0.5	$\infty$	$\infty$
1.5	9.9	14.8	0.7	19	13.3	0.2	21.4	4.3	1.0	14.7	14.7
2	6.8	13.6	1.0	13.3	13.3	0.3	15	4.5	1.5	9.75	13.6
2.5	4.9	12.2	1.2	11.5	13.8	0.4	10.9	4.4	2.0	6.5	13.0
3	4.2	12.6	1.5	9.3	13.9	0.6	7	4.2	2.5	5.5	13.7
						0.7	6.8	4.8	3.0	4.25	12.7
						1.0	4.6	4.6	---	---	---

It is very important, however, for the correct interpretation of the results which follow to bear in mind the relative values of the reduction times for, say, four donators, formic acid, lactic acid, succinic acid and glucose, with any particular organism, and the change in these values as the concentration of organism is changed (see Table II). The ratio of reduction times

$$t_{\text{formic acid}} : t_{\text{lactic acid}} : t_{\text{succinic acid}} : t_{\text{glucose}}$$

is always of the same order for a normal organism (*B. coli*) so long as the reduction time in presence of *M*/140 formic acid is not too great (not greater than 20'). Thus, experience has shown us that with any normal organism (*B. coli*) prepared in the way we have described, if  $t_{\text{formic acid}} = 3'$  to  $7'$  then  $t_{\text{succinic acid}}$  will be about  $14'$ , and  $t_{\text{glucose}}$  about  $7'$  and so forth. Any great deviations from this order, which are produced by a particular factor, must be due to the relatively greater action of this factor on some mechanisms than upon others; they cannot be interpreted as due to the inactivation of the organism as a whole. For instance, if for the normal organism we have

$$t_{\text{formic acid}} = 4; t_{\text{succinic acid}} = 14; t_{\text{glucose}} = 6,$$

and on treating this organism in a certain way we now find

$$t_{\text{formic acid}} = 5; t_{\text{succinic acid}} = 70; t_{\text{glucose}} = \infty,$$

this must mean that the mechanism which activates glucose is relatively more sensitive to the treatment than that which deals with succinic acid and similarly the latter is more sensitive than that which deals with formic acid. The effect cannot be interpreted as due to the inactivation of a certain proportion of the organisms *as a whole*. If, on the other hand, we find after treatment

$$t_{\text{formic acid}} = 20; t_{\text{lactic acid}} = 50; t_{\text{succinic acid}} = 120; t_{\text{glucose}} = 50,$$

or

$$t_{\text{formic acid}} = 40; t_{\text{lactic acid}} = 120; t_{\text{succinic acid}} = \infty; t_{\text{glucose}} = \infty,$$

this would indicate the improbability of a selective action and point to an inactivation of a large proportion of the organisms as a whole.

Table II.

*Coli* 4. Conditions as in Table I.

Reduction times with varying concentrations of organism.

$\infty$  = not reduced in 6 hours.

<i>q</i>	...	...	1	1/2	1/4	1/8	1/16	1/32
Succinic acid <i>M</i> /140			17	36.5	77	390	$\infty$	$\infty$
Lactic acid <i>M</i> /140			8	15	34	126	250	$\infty$
Formic acid <i>M</i> /140			3.2	4.5	10	18	42	91
Acetic acid 3 <i>M</i> /14			4	6	12.5	24	55	164
$\alpha$ -Hydroxybutyric acid <i>M</i> /70			10	20	58	$\infty$	$\infty$	$\infty$
*Glycerol <i>M</i> /7			11.5	19	138	$\infty$	$\infty$	$\infty$
Glutaminic acid 3 <i>M</i> /140			17	37	79	$\infty$	$\infty$	—
Glucose <i>M</i> /140			6.5	13	27	201	$\infty$	$\infty$
Sorbitol <i>M</i> /140			10	23.5	77	$\infty$	$\infty$	$\infty$
Growth after 18 hours from one loopful of the suspension on agar			—	—	Prolific	Prolific	Prolific	Prolific

\* In the case of glycerol, 1 cc. 5 % NaCl was added to the vacuum tubes.

## II. The effect of exposure of *B. coli* to varying temperatures.

To 40 cc. of the *B. coli* suspension, 10 cc. of a phosphate (Clark and Lubs) buffer solution  $p_H$  7.4 were added. The suspension was placed in a thermostat kept at the required temperature for 1 hour after it had attained this temperature. It was then surrounded by ice and the activations of the organism were determined in the usual way. For the low temperature work the organism

was placed for 1 hour in a cold room kept at the required temperature. In the case of the liquid air experiments the suspension was placed in a large test-tube and slowly immersed into liquid air. It was then withdrawn and placed immediately in another bath of liquid air for 1 hour. After transferring the tube to a beaker it was allowed to thaw at 37° and finally, on thawing, the beaker was cooled in ice. The activations of the organism were then determined. In the case of the organism exposed to 77° coagulation of the organism was observed to occur at 75°. No coagulation at lower temperatures was observed.

Results with these heated or cooled organisms are noted in Table III.

Table III.

*Coli* 5. Conditions as in Table I.  
Reduction times after exposure to varying temperatures.

Temperature to which the organism was exposed ...	... -180°	-21°	-6°	37°	47°	57°	67°	77°
Succinic acid <i>M</i> /140	20	19	16.7	16.2	20	24	∞	—
Lactic acid <i>M</i> /140	9	6.5	6	6	6.7	83	∞	—
Formic acid <i>M</i> /140	7	5.5	5.7	6	5.5	7	18	∞
Acetic acid <i>M</i> /4.6	12.5	8.7	8.2	7.5	8	11.7	26	∞
Butyric acid <i>M</i> /4.6	10	10.5	8	8.25	10.7	10.5	27	∞
α-Hydroxybutyric acid <i>M</i> /70	36	9.5	10	7.7	16.8	∞	∞	∞
Alanine <i>M</i> /14	—	—	—	66	∞	∞	—	—
Glutaminic acid <i>M</i> /140	—	—	—	31	63.5	∞	—	—
Glycol <i>M</i> /4.6	∞	—	—	5.5	36	∞	∞	∞
Glycerol <i>M</i> /7	∞	—	—	29.5	69.5	∞	—	—
α-Glycerophosphoric acid <i>M</i> /7	8	6	5.7	5	6.7	11.5	26.5	∞
Glucose <i>M</i> /140	∞	9	9.5	7.3	15	∞	∞	∞
Fructose <i>M</i> /140	∞	7.5	6.7	5.9	12	∞	∞	∞
Mannose <i>M</i> /140	∞	11.25	10.25	8	14.5	∞	—	—
Galactose <i>M</i> /140	∞	10	12	7.7	16	∞	—	—
Mannitol <i>M</i> /140	∞	17.5	15	11.5	27	∞	∞	∞
Sorbitol <i>M</i> /140	∞	22.5	17.7	13.5	35	∞	—	—
Fumaric acid <i>M</i> /35*	+	—	—	+	+	+	0	—
Na nitrate <i>M</i> /70*	+	—	—	+	+	+	0	—
K chlorate <i>M</i> /70*	+	—	—	+	+	+	0	—

\* A + sign indicates that activation occurs, a zero sign that it has disappeared. The activation is determined by the inability of a mixture of the substance with an active donator to reduce methylene blue in presence of the organism.

Examination of the table shows that change in temperature has a selective action on the activating mechanisms of the organism. Thus we have for exposure at 37° the normal values:

$$t_{\text{formic acid}} = 6; t_{\text{succinic acid}} = 16.2; t_{\text{lactic acid}} = 6; t_{\text{glucose}} = 7.3.$$

For exposure at 57° we obtain

$$t_{\text{formic acid}} = 7; t_{\text{succinic acid}} = 24; t_{\text{lactic acid}} = 83; t_{\text{glucose}} = \infty.$$

As the temperature is increased we find that the most sensitive mechanisms are those for alanine, glycerol, glycol, the sugars and glutaminic acid; then come those for lactic acid, succinic acid and fumaric acid, the most resistant being those for formic, acetic, butyric, and α-glycerophosphoric acids.

Exposure of the organism for an hour to a temperature of  $-21^{\circ}$  does not appear to have an appreciable effect, but exposure to the temperature of liquid air for this time affects the organism considerably. Here again the most sensitive mechanisms are those for glycol, glycerol, the sugars and glutaminic acid. The other mechanisms are but slightly affected.

### III. *The effect of exposure of B. coli to varying H ion concentrations.*

To 45 cc. of the suspension of *B. coli* were added 25 cc. of a buffer solution at the required  $p_H$  and 10 cc. of normal saline solution. The suspension was warmed to  $37^{\circ}$  and then placed in the incubator at  $37^{\circ}$  for 1 hour. It was centrifuged, washed with normal saline, emulsified and made up to 45 cc. with saline. The activations of the treated organism were then determined. Phthalate buffers were used for  $p_H$  2.2, 3.0, 3.8 and 5.0; phosphate buffer for  $p_H$  7.0 and borate for  $p_H$  9.0 and 11.0. In one series no buffer, but caustic soda to reach a final concentration of  $N/30$  was added. At  $p_H$  2.2, 3.0 and 3.8 the organism was flocculated and difficult to emulsify after centrifuging; between  $p_H$  5 and 11 there was no apparent change in the organism. According to Nicolai [1926] the isoelectric point of *B. coli* is at  $p_H$  4.5. In  $N/30$  NaOH the organism appeared to be partially dissolved. The results are expressed in Table IV.

Table IV.

*Coli* 6. Conditions as in Table I.  
Reduction times after exposure to varying H ion concentrations.

$p_H$ at which the organism was exposed	...	2.2	3.0	3.8	5.0	7	9	11	that of $N/30$ NaOH
Succinic acid $M/140$		$\infty$	$\infty$	83	45	14	15	43	$\infty$
Lactic acid $M/140$		$\infty$	$\infty$	23.5	10.3	7	6	13	$\infty$
Formic acid $M/140$		81	16	5.1	5	6	5.1	14.5	$\infty$
Acetic acid $M/14$		61	17	15.5	6.6	6	6	22	$\infty$
Butyric acid $M/4.6$		58	14	8.5	5.8	6.7	6	16.5	$\infty$
$\alpha$ -Hydroxybutyric acid $M/70$		$\infty$	$\infty$	66	11.3	6.5	8.7	27.5	$\infty$
Alanine $M/14$		$\infty$	$\infty$	$\infty$	42	28.3	19	$\infty$	—
Glutaminic acid $M/140$		$\infty$	$\infty$	$\infty$	81	34.3	23	$\infty$	—
Glycol $M/4.6$		$\infty$	$\infty$	90	51	15.3	20	90	$\infty$
Glycerol $M/7$		$\infty$	$\infty$	110	76	20.2	24.7	$\infty$	—
$\alpha$ -Glycerophosphoric acid $M/7$	108	13	6.2	5	5.1	4.2	14	$\infty$	$\infty$
$\beta$ -Glycerophosphoric acid $M/7$	$\infty$	$\infty$	$\infty$	$\infty$	31	27	$\infty$	—	—
Tartaric acid $M/4.6$	$\infty$	$\infty$	$\infty$	$\infty$	34	19	25	$\infty$	$\infty$
Glucose $M/140$	$\infty$	$\infty$	16	6.7	5.2	5.2	36	$\infty$	$\infty$
Fructose $M/140$	$\infty$	$\infty$	16	4.5	5	5.2	28.5	$\infty$	$\infty$
Mannose $M/140$	$\infty$	$\infty$	23.7	5.5	4.7	6.9	33	$\infty$	$\infty$
Galactose $M/140$	$\infty$	$\infty$	17	5.7	4.8	6.3	32	$\infty$	$\infty$
Xylose $M/140$	$\infty$	$\infty$	$\infty$	19	5.25	8	$\infty$	$\infty$	$\infty$
Mannitol $M/140$	$\infty$	$\infty$	27	7.7	9.7	9.2	43	$\infty$	$\infty$
Sorbitol $M/140$	$\infty$	$\infty$	23.5	8	8	9.2	42	$\infty$	$\infty$
Fumaric acid $M/35$	0	0	+	+	+	+	+	—	—
Na nitrate $M/70$	0	0	+	+	+	+	+	—	—
K chlorate $M/70$	0	0	+	+	+	+	+	—	—

It will be observed that exposure to different hydrogen ion concentrations has a selective effect on the activating mechanisms of the organism.



The mechanisms most sensitive to increase in hydrogen ion concentration are those for glycerol, glycol, glutaminic acid and  $\beta$ -glycerophosphoric acid; then come those for the sugars and succinic acid; then that for lactic acid, and finally, the most resistant, those for formic acid, acetic acid, butyric acid and  $\alpha$ -glycerophosphoric acid. Increase in H ion concentration has not so vigorous an effect on the sugar-activating mechanisms relative to those for succinic acid and lactic acid as has increase in temperature.

#### IV. *The effect of exposing B. coli to varying salt concentrations.*

To 70 cc. of a suspension of *B. coli* were added 40 cc. buffer solution of the appropriate  $p_H$ , the required quantity of pure sodium chloride dissolved in distilled water and sufficient water to make up the volume to 150 cc. The suspension was warmed to 37° and then incubated for 1 hour or for 24 hours at 37°. After the period of treatment the organism was washed by centrifuging, emulsified and made up to 70 cc. with normal saline solution. Its activations were determined and are noted in Table V. With the strain used for the work expressed in Table V only exposure to a concentration of 14 % NaCl produced a flocculation of the organism. It was easy to emulsify the centrifuged deposit of the treated organism.

Examination of the table shows that exposure to high salt concentrations results in a selective destruction of the activating mechanisms. Thus, whereas exposure at  $p_H$  7.4 for 1 hour to 5 % NaCl has relatively little effect,

$$t_{\text{formic acid}} = 4.5; t_{\text{lactic acid}} = 7.5; t_{\text{succinic acid}} = 10; t_{\text{glucose}} = 8.7,$$

exposure at  $p_H$  7.4 for 24 hours to 5 % NaCl gives us

$$t_{\text{formic acid}} = 6.2; t_{\text{lactic acid}} = 7.7; t_{\text{succinic acid}} = 18; t_{\text{glucose}} = \infty.$$

The effect of time on the action of high concentrations of salts is very marked. The mechanisms most sensitive are, as in previous cases, those for glycerol, glutaminic acid and the sugars. Then come those for succinic acid and lactic acid, and the most resistant are those for formic acid, acetic acid, butyric acid and  $\alpha$ -glycerophosphoric acid.

An interesting point is the distinction between fructose and glucose shown in the third and fifth columns of the table. We will notice this distinction in a number of other cases.

#### V. *The effect of exposure of B. coli to varying concentrations of nitrites.*

The treatment was carried out here exactly in the same way as that described in section IV. The results are expressed in Table VII, comparative results with similar concentrations of sodium chloride being also given. Columns K and J of Table X show results carried out with another strain of *B. coli* exposed to 5 % NaCl and to 5 % NaNO<sub>2</sub> at  $p_H$  6.0 for 1 hour. Table VI gives results, less systematically carried out, with a different strain of *B. coli* exposed to nitrites under various conditions.

Table V.

*Coli* 7. Conditions as in Table I. Temperature of exposure 37°. Reduction times after exposure to varying salt concentrations.

Conc. of NaCl to which the organism was exposed ...	Control Normal organism	5 %	5 %	5 %	5 %	14 %	14 %	5 %	5 %
Time of exposure in hrs. ...	—	1	24	1	24	1	24	1	24
<i>p<sub>H</sub></i> ...	7.4	5.0	5.0	7.4	7.4	7.4	7.4	9.0	9.0
Succinic acid <i>M</i> /140	9.5	23.5	40	10	18	14.5	17	22	27.5
Lactic acid <i>M</i> /140	5.7	9.5	11	7.5	7.7	7	13	11	33
Formic acid <i>M</i> /140	4.5	6	4	4.5	6.2	4	5.5	7	6.5
Acetic acid <i>M</i> /4.6	4.5	6.5	4.5	5	5	5	6.5	6.2	10.2
Butyric acid <i>M</i> /4.6	5	6	6	5.5	7	7	6.5	6.5	9.3
$\alpha$ -Hydroxybutyric acid <i>M</i> /70	7.5	14.5	35.7	6.5	19.5	10	24.5	13.2	105
$\alpha$ -Glycerophosphoric acid <i>M</i> /14	4	6	4	5	6	6	5	5	11
Glycerol <i>M</i> /7	14	$\infty$	$\infty$	25	$\infty$	$\infty$	$\infty$	$\infty$	$\infty$
Glutaminic acid <i>M</i> /46	12.5	24	$\infty$	21.5	$\infty$	41	$\infty$	105	$\infty$
Glucose <i>M</i> /140	6.5	9.2	$\infty$	8.7	$\infty$	16.5	$\infty$	15	$\infty$
Fructose <i>M</i> /140	5	8	114	6.5	31	9.2	$\infty$	11.7	$\infty$
Mannose <i>M</i> /140	7	10	$\infty$	9.5	$\infty$	12.5	$\infty$	17.5	$\infty$
Galactose <i>M</i> /140	6.5	14	$\infty$	8.5	$\infty$	13.2	$\infty$	18	$\infty$
Mannitol <i>M</i> /140	9.2	17.5	$\infty$	12	$\infty$	20.5	$\infty$	27	$\infty$
Sorbitol <i>M</i> /140	10	17.5	$\infty$	10.5	$\infty$	19	$\infty$	25	$\infty$
Fumaric acid <i>M</i> /35	+	+	+	+	+	+	+	+	+
Na nitrate <i>M</i> /70	+	+	+	+	+	+	+	+	+
K chlorate <i>M</i> /70	+	+	+	+	+	+	+	+	+

Table VI.

Conditions as in Table I. Temperature of exposure 37°.

<i>Coli</i>	Concentration of NaNO <sub>2</sub> to which the organism was exposed o/o	<i>p<sub>H</sub></i> of exposure	Time of exposure hrs.	<i>t</i> <sub>glucose</sub> mins.	<i>t</i> <sub>succinic acid</sub> mins.	<i>t</i> <sub>lactic acid</sub> mins.	<i>t</i> <sub>formic acid</sub> mins.
9	1	6.8	22	60	$\infty$	12.5	3.8
9	1	6.8	46	$\infty$	$\infty$	18	26
10	5	7.0	20	$\infty$	$\infty$	60	8
10	2	6.3	24	—	$\infty$	33	54
11	1	6.8	30	—	$\infty$	19	58

It will be observed that the tendency of the effect of nitrites is towards selectivity of action, though this is not always well marked. Nitrites at a low *p<sub>H</sub>* are more destructive than those at a higher *p<sub>H</sub>*, as would be anticipated if nitrous acid were the destructive agent. The very clear difference between the effects of nitrites and those of NaCl under the same conditions shows that the action of the former is not entirely an osmotic one.

As to selectivity of action, that due to nitrites seems to be similar in its general tendency to the action brought about by exposure to increased hydrogen ion concentrations (see Table IV). If indeed an amino-group plays an essential part in the activations, this similarity of action is to be anticipated.

Table VII.

*Coli* 8. Conditions as in Table I, except that in columns *A* and *B* each vacuum tube contained 1 cc. 5 % NaCl. In every case in this table, as in Tables VIII, IX and X, the glycerol tube contained 1 cc. 5 % NaCl. This was found to be beneficial, with glycerol, in accelerating the last stage of the reduction. Temperature of exposure 37°.

Reduction times after exposure to varying NaCl and NaNO<sub>2</sub> concentrations.

Character of exposure ...	<i>A</i> Control Normal organism	<i>B</i> 5 % NaCl	<i>C</i> 2 % NaCl	<i>D</i> 2 % NaNO <sub>2</sub>	<i>E</i> 1 % NaCl	<i>F</i> 1 % NaNO <sub>2</sub>	<i>G</i> 1 % NaCl	<i>H</i> 1 % NaNO <sub>2</sub>	<i>J</i> 1 % NaCl and 1 % H.COONa	<i>K</i> 1 % NaNO and 1 % H.COONa
Time of exposure in hrs. ...	—	1	1	1	1	1	23	23	23	23
<i>p<sub>H</sub></i> of exposure ...	—	5.0	5.0	5.0	6.0	6.0	6.0	6.0	6.0	6.0
Succinic acid <i>M</i> /140	17	121	60	∞	16.2	∞	22	∞	55	∞
Lactic acid <i>M</i> /140	8.7	47	18	107	8.7	24	11	∞	16.5	77
Formic acid <i>M</i> /140	4	5	4	27	3	4.2	3.2	52	5	∞
Acetic acid <i>M</i> /46	4.7	5.7	3.2	27.5	3.7	5.7	4	∞	5.7	∞
Butyric acid <i>M</i> /46	6	7	6	41	4.2	6.7	5	∞	6	∞
α-Hydroxybutyric acid <i>M</i> /70	10	35	17	∞	10.5	34.2	20.7	∞	36.2	∞
α-Glycerophosphoric acid <i>M</i> /14	11	13.5	6	∞	5.5	7	4.2	—	7.2	∞
Glycerol <i>M</i> /7	13.5	80	29.5	∞	27	46	46.2	∞	76	∞
Glutamic acid <i>M</i> /46	21.2	∞	85	∞	16.5	125	61	∞	∞	—
Glucose <i>M</i> /140	7	15	9.5	∞	7.2	14.2	13	∞	24	∞
Fructose <i>M</i> /140	7.5	18	8.5	∞	5.2	15.5	10	∞	21	∞
Mannose <i>M</i> /140	8	17	8	—	6	15.7	13.2	—	30.5	—
Galactose <i>M</i> /140	7	15.7	10	—	6.5	15.5	12	—	26	—
Mannitol <i>M</i> /140	11.2	41	17	∞	8	20	18.5	∞	34	—
Sorbitol <i>M</i> /140	10.5	28.5	17.5	—	10	25.7	20	—	37	—
Fumaric acid <i>M</i> /35	—	—	—	—	+	0	—	—	—	—
Na nitrate <i>M</i> /70	—	—	—	—	+	+	—	—	—	—
K chlorate <i>M</i> /70	—	—	—	—	+	+	—	—	—	—

We have not observed any protective action by formates on the formic acid-activating mechanism in *B. coli* when this is exposed to a mixture of nitrites and formates. A protective action has been found by Myrbäck [1926] in the case of invertase, by the presence of sucrose, when this enzyme is exposed to nitrous acid. Inspection of columns *B* and *C* of Table IX and of column *K* of Table VII shows that no such protective action by formates occurs, under our experimental conditions, with *B. coli*.

On exposure of the organism, *coli* 8, to 2 % NaCl at *p<sub>H</sub>* 5.0, and to 1 % NaCl at *p<sub>H</sub>* 6.0 flocculation of the organism occurred. Under the same conditions no appreciable flocculation occurred when NaNO<sub>2</sub> was substituted for NaCl; a slight flocculation was observable only in the case of exposure for 23 hours to 1 % NaNO<sub>2</sub> at *p<sub>H</sub>* 6.0. Since the effects of the same concentration of salt on different strains vary somewhat, quantitatively, it is necessary when observing the effects of nitrites to perform comparative experiments with equivalent amounts of salt.

#### VI. The effect of methylene blue on *B. coli*.

To 12 cc. of the suspension of *B. coli* were added 12 cc. phosphate buffer *p<sub>H</sub>* 7.4 and 12 cc. 1/5000 methylene blue solution. The suspension was allowed to stand in a thermostat at 45° for 1½ hours. After this period it was cooled in ice and 3 cc. of the mixture were added to a vacuum tube containing 1 cc. phosphate buffer *p<sub>H</sub>* 7.4, 1 cc. of the donator solution and 2 cc. normal saline. The reduction times for the methylene blue were then determined at 45° in the usual way. The following results were obtained:

$$t_{\text{succinic acid}} = \infty; t_{\text{lactic acid}} = 43; t_{\text{formic acid}} = 7;$$

$$t_{\text{glucose}} = 50; t_{\text{sorbitol}} = 52.$$

For *B. coli* treated similarly but in the absence of methylene blue we obtained:

$$t_{\text{succinic acid}} = 18; t_{\text{lactic acid}} = 9; t_{\text{formic acid}} = 4;$$

$$t_{\text{glucose}} = 6; t_{\text{sorbitol}} = 9.2.$$

Table VIII.

*Coli* 14. Conditions as in Table I.  $p_H$  of exposure = 7.4.

	Reduction times.										
	A	B	C	D	E	F	G	H	J	K	L
Character of exposure	Control Normal organism		10 % cyclo- hexanol	10 % cyclo- hexanol	Ether	"Col- loidal iron"	"Col- loidal iron" and toluene	Methy- lene blue at 45°	Control on H at 45°	15 % CHCl <sub>3</sub>	15 % CHCl <sub>3</sub>
Time of exposure	...	—	5 mins.	5 mins.	10 mins.	5 mins.	5 mins.	2 hrs.	2 hrs.	30 mins.	18 hrs.
			Not re- moved	Re- moved							
Succinic acid M/140	9.2	10.2	—	∞	16	14	16.7	163	20	59	80
Lactic acid M/140	5.7	6.7	∞	∞	11.2	10	16	49	12	11	14.5
Formic acid M/140	3	3	∞	15.2	3.7	4.5	5.5	5.5	4.2	6.2	11
Acetic acid M/4.6	3.7	4.5	∞	49.5	6	5.2	8.5	8	6.2	8.7	8.7
Butyric acid M/4.6	5.7	5.2	∞	28	5.7	6.2	5	6.5	5.7	—	—
α-Hydroxybutyric acid M/70	7.7	23.5	—	∞	40	13	44	∞	25	51	70
α-Glycerophosphoric acid	3.7	4.5	∞	14.5	5	5.2	8	—	—	7	7
β-Glycerophosphoric acid	6	∞	∞	66	—	—	—	—	—	—	—
Glycerol M/7	10	∞	—	∞	∞	17	∞	∞	29	∞	∞
Glutaminic acid M/46	11.7	∞	—	∞	∞	19	∞	∞	31.5	∞	∞
Glucose M/140	4.5	∞	—	∞	∞	8	∞	∞	10	∞	∞
Fructose M/140	4	107	—	∞	129	8.5	∞	∞*	10.5	115	∞
Mannose M/140	4.2	∞	—	∞	∞	9.5	∞	∞	11.5	∞	∞
Galactose M/140	4.5	∞	—	∞	∞	8	∞	∞	13.2	∞	∞
Mannitol M/140	9.5	∞	—	∞	∞	16	∞	∞	16	∞	∞
Sorbitol M/140	7	∞	—	∞	∞	17	∞	∞	21	∞	∞
Fumaric acid M/35	—	+	—	0	+	+	—	+	—	+	+
Na nitrate M/70	—	+	—	+	+	+	—	+	—	+	+
K chlorate M/70	—	+	—	+	+	+	—	+	—	+	+
Growth on agar from a loopful of treated organism	Prolific	Few discrete colonies	—	Few discrete colonies	Few discrete colonies	Prolific	Few discrete colonies	Few discrete colonies	Prolific	None	—

\* This tube reduced completely in 5 hours whereas the other sugars showed no reduction in this time.

Table VIII, column H, gives results with another strain of *B. coli* treated for 2 hours with methylene blue in a similar manner to the above. The results with the organism treated similarly but in the absence of the dyestuff are given in column J.

It will be observed that a selectivity of action is demonstrable. As before, the most resistant mechanisms are those for formic acid, acetic acid, and butyric acid. The sugars, glycerol, glutaminic acid and succinic acid systems are among the least resistant. The organism treated with methylene blue on subculture gave rise to a few discrete colonies, whereas the control gave a prolific growth.

#### VII. The effects of toluene, benzene and phenol on *B. coli*.

To 25 cc. of the suspension of *B. coli* were added 5 cc. phosphate buffer solution  $p_H$  7.4 and 5 cc. toluene. The mixture was shaken, allowed to stand for 5 minutes at room temperature and then centrifuged. The deposit of organism was washed and made up to 25 cc. with normal saline solution. The organism was treated in the same way with benzene. Results with

Table IX.

Coli 12. Conditions as in Table I. Concentrations of substrates as in Table VIII.

Character of exposure ...	Time of exposure ...	Reduction times.									
		A	B	C	D	E	F	G	H	J	K
		Control Normal organism	1 % NaCl + 1 % NaNO <sub>2</sub>	1 % H.COONa + 1 % NaNO <sub>2</sub>	1 % NaCl + 1 % H.COONa	1 % H.COONa	Toluene	C <sub>2</sub> H <sub>5</sub> OH	CHCl <sub>3</sub>	cyclo- Hexanol	cyclo- Hexanol
		—	17 hrs. pH 7.0	17 hrs. pH 7.0	17 hrs. pH 7.0	17 hrs. pH 7.0	5 mins	30 mins.	4 hrs.	Not re- moved	Re- moved after 5 mins.
Succinic acid	14.2	∞	∞	∞	34	32.5	21.5	∞	69	∞	∞
Lactic acid	7.7	54	41.5	17	13.5	8.7	25	10	∞	∞	13
Formic acid	4	14.2	∞	7.2	6.2	4.7	25.7	9.2	∞	49	21.5
Acetic acid	5.5	15.2	∞	7.5	7	6.5	19.7	20.2	∞	100	22.5
Butyric acid	7.5	20.7	∞	9.2	8.5	7	21	15.5	∞	—	61
α-Hydroxybutyric acid	10.5	∞	114	25	28	45	∞	38	∞	—	∞
α-Glycerophosphoric acid	5.5	37	∞	9	8.5	7.2	20	9.5	∞	—	14
β-Glycerophosphoric acid	9.2	—	—	—	—	∞	—	—	—	—	—
Glycerol	11.2	∞	∞	19.2	29	∞	∞	∞	∞	—	—
Glutaminic acid	14.5	72	—	—	—	∞	∞	∞	∞	—	—
Glucose	5.2	55	114	9	13	∞	∞	∞	∞	∞	—
Fructose	5.5	—	69	10.5	8	∞	∞	∞	∞	∞	—
Mannose	—	—	—	—	—	∞	∞	∞	∞	—	—
Galactose	—	—	—	—	—	∞	∞	∞	∞	—	—
Mannitol	7.2	∞	115	—	12.5	∞	∞	∞	∞	—	—
Sorbitol	—	—	—	—	—	∞	∞	∞	∞	—	—
Fumaric acid	—	?	0	—	—	+	0	+	—	—	—
Na nitrate	—	+	+	—	—	+	+	+	—	—	—
K chlorate	—	—	—	—	—	+	+	+	—	—	—

Table X.

Coli 15. Conditions as in Table I. pH of exposure 7.4. Concentrations of substrates as in Table VIII.

Character of exposure ...	Time of exposure ...	Reduction times.								
		A	B	C	D	E	F	G	H	K
		Coli 12 Ether	Thorough extrac- tion with ether	1 % C <sub>2</sub> H <sub>5</sub> OH	7 % C <sub>2</sub> H <sub>5</sub> OH	14 % C <sub>2</sub> H <sub>5</sub> OH	15 % benzene	21 % acetone	1 % phenol	5 % NaCl pH 6.0
		30 mins.	—	5 mins.	5 mins.	30 mins.	1 min.	60 mins.	5 mins.	5 % NaNO <sub>2</sub> pH 6.0
Succinic acid	11.5	23.5	11.7	27.7	42	12.7	20	44	16.2	∞
Lactic acid	11.2	12	6.5	9.2	8.5	9	9	7.6	11	34
Formic acid	4.5	3	2.5	3.2	3.2	2.5	2.7	4.2	3.5	7.5
Acetic acid	5.7	3.2	—	4.2	4	3	3.7	6	4.2	17
Butyric acid	7.7	3.7	—	5	5	5	4.2	3.7	4	16.5
α-Hydroxybutyric acid	58	27	—	21.5	46	20.7	19.5	34.5	12.7	80
α-Glycerophosphoric acid	7.7	3.7	—	4.7	4.7	3.5	4.5	4.7	5.2	43
β-Glycerophosphoric acid	110	∞	—	117	∞	—	∞	∞	—	∞
Glycol	—	—	—	—	—	—	∞	∞	65	∞
Glycerol	∞	∞	—	58	∞	∞	∞	∞	15	∞
Glutaminic acid	∞	∞	—	14.5	∞	∞	∞	∞	20.5	∞
Glucose	∞	∞	5.2	15	∞	∞	41.5	∞	7	∞
Fructose	161	101	—	11	∞	67	15	147	7.2	124
Mannose	∞	∞	—	11.5	∞	∞	58	∞	6.5	∞
Galactose	∞	∞	—	11.5	∞	∞	60	∞	8	∞
Mannitol	∞	∞	—	19.5	∞	∞	∞	∞	15.5	∞
Sorbitol	∞	∞	—	20.5	∞	∞	∞	∞	16	∞
Fumaric acid	+	+	—	—	—	+	—	+	—	0
Na nitrate	+	—	—	—	—	+	—	+	—	+
K chlorate	+	—	—	—	—	+	—	+	—	+
Growth on agar from a loopful of the treated organism	—	—	Prolific	Prolific	Few discrete colonies	None	Good	Few discrete colonies	—	—

toluene are given in column *B*, Table VIII, and column *F*, Table IX those with benzene are given in column *F*, Table X.

The most striking effect of these substances after 5 minutes' treatment is the elimination of the mechanisms dealing with the sugars (though that dealing with fructose shows relatively considerable resistance), with glycerol and with glutaminic acid. The mechanisms for succinic, lactic, formic, acetic, butyric and  $\alpha$ -glycerophosphoric acids appear to be left almost intact. Subculture of the treated organism shows that the treatment has been practically completely lethal—only a few discrete colonies are obtained.

In the case of phenol, 25 cc. of the suspension of *B. coli* were treated with 1 % neutralised phenol in presence of phosphate buffer  $p_H$  7.4 for 5 minutes at room temperature, centrifuged and washed with saline. The washed organism was made up to 25 cc. with normal saline. Its activations are noted in column *H* of Table X. It will be observed that the same order of sensitivity obtains as with benzene and toluene; the succinic acid mechanism appears to be somewhat affected. Subculture shows that the treatment has been almost completely lethal.

#### VIII. *The effects of ether and chloroform on B. coli.*

*B. coli* was treated with ether and chloroform in the same way as described for benzene and toluene. The time of treatment with ether was 10 minutes (column *E*, Table VIII) and with chloroform 30 minutes (column *K*, Table VIII) and 18 hours (column *L*, Table VIII). With another strain (*coli* 12) the time with ether was 30 minutes (column *A*, Table X) and with chloroform 4 hours (column *H*, Table IX) and 24 hours (column *L*, Table IX). The same effects as as with toluene and benzene are obtained. It is apparent that with lapse of time the more resistant mechanisms disappear.

The following experiment was carried out with ether. 100 cc. of a thick freshly prepared suspension of *B. coli* were extracted three times with ether. The ether extracts were collected, and evaporated to dryness *in vacuo* at 20°. The activations of the extracted organism, which was washed twice with normal saline, are noted in column *B*, Table X. Half of the quantity of the extracted organism was added to the vessel containing the slight residue from the ether extract and the suspension was incubated at 37° for 2 hours. The activations of the incubated organism for succinic acid and glucose were then determined. It was found that the mechanism for glucose was still absent and that for succinic acid had disappeared. The other half of the quantity of extracted organism was allowed to stand in the incubator at 37° for 16 hours after which time it was found that the succinic acid mechanism had been eliminated.

#### IX. *The effect of acetone on B. coli.*

If a suspension of *B. coli* be shaken with small concentrations of acetone, no appreciable effects occur. If the acetone concentration be increased, selective

effects become apparent. Column *G*, Table X, gives results obtained with *B. coli* treated with 21 % acetone.

The results with acetone are interesting in showing a selective behaviour with the sugars. Thus the mechanism for fructose is most resistant, as is usually the case, whilst those for glucose, mannose and galactose are more resistant than those for the polyhydric alcohols. On the whole the effects of high concentrations of acetone are similar to those of chloroform, ether, etc.

#### X. *The effects of propyl alcohol and cyclo-hexanol on B. coli.*

It has been shown by Quastel and Whetham [1925, 1, 2] that the presence of propyl alcohol in the vacuum tube at a concentration of 20 % will prevent any reduction of methylene blue by donators in presence of *B. coli*. Similarly, cyclo-hexanol has a powerful inhibiting action on reductions in the presence of *B. coli*. It was interesting to see whether this action of propyl alcohol and cyclo-hexanol was reversible, *i.e.* whether after exposure of *B. coli* to such concentrations of alcohol, which in the vacuum tubes are entirely inhibitive to all reductions, activations can still occur.

To 50 cc. of *B. coli* suspension were added 20 cc. propyl alcohol and the suspension shaken and allowed to stand at room temperature for 30 minutes. (This concentration of propyl alcohol in the vacuum tube is sufficient entirely to inhibit reductions due to any of the donators we use.) Flocculation of the organism occurred. It was washed by centrifuging and suspended in 50 cc. normal saline. The activations of the treated organism are shown in column *G*, Table IX. It will be observed that there is reversibility with the mechanisms dealing with lactic, formic, acetic, butyric and  $\alpha$ -glycerophosphoric acids.

The effects of exposing *B. coli* to 1 % propyl alcohol for 5 minutes with subsequent washing are shown in column *C*, to 7 % propyl alcohol for 5 minutes in column *D*, and to 14 % propyl alcohol for 30 minutes in column *E* of Table X. Exposure to the last concentration of alcohol resulted in elimination of the activation of glycerol, the sugars and glutaminic acid; at this concentration, too, there was an almost completely lethal action, for subculture showed but few colonies. Exposure to the previous two concentrations which did not affect the sugar activations appreciably had but little lethal action, for subculture yielded prolific growths. Although exposure to 7 % propyl alcohol with subsequent removal of the alcohol did not result in elimination of the glucose-activating mechanism, the same concentration in the vacuum tube entirely inhibited reduction due to glucose. Thus a reversibility in the case of glucose is apparent.

The partially reversible action with propyl alcohol is also exhibited by cyclo-hexanol. Column *C*, Table VIII, and column *J*, Table IX, give results with 10 % cyclo-hexanol present in the vacuum tube; column *D*, Table VIII, and column *K*, Table IX, show the results obtained after exposure of *B. coli* to the cyclo-hexanol for 5 minutes with subsequent washing. The effect of cyclo-hexanol is more vigorous and less easily reversible than that of propyl alcohol.

XI. *The effect of "dialysed iron" on B. coli.*

10 cc. of commercial "dialysed iron"<sup>1</sup> was diluted to 100 cc. with distilled water, 2 drops of dilute ammonia added and the solution filtered, the filtrate now having a neutral reaction. 10 cc. of this solution were added to 50 cc. of a suspension of *B. coli* and the mixture was allowed to stand 1 hour, at the end of which time the organism had entirely flocculated, adsorbing the "iron." It was centrifuged and the deposit washed. The washed deposit was suspended in 50 cc. saline. The activations of the treated organism are noted in column *F*, Table VIII, and it will be observed that there has been but little interference by the adsorbed "iron" with the activations of the organism. That the adsorbed "iron" does not protect the organism appreciably is shown by the following experiment. 20 cc. of the "iron"-treated organism were treated with toluene in the manner described earlier. After washing, its activations were determined and are noted in column *G*, Table VIII. The effects of toluene on the treated organism are the same as those in the absence of adsorbed "iron" on the organism.

XII. *The action of sodium hydrosulphite.*

We have now made a number of attempts to restore the activating mechanisms of *B. coli* which have been eliminated by a particular treatment. Though partial reversibility occurs in the cases of propyl alcohol and cyclohexanol we have had but little success with the more deep-seated changes which occur with other agents.

An effect, however, with sodium hydrosulphite on the nitrite-treated organism is curious and although it has not yet been fully investigated it is, we think, worthy of description here.

To 175 cc. of a suspension of *B. coli* were added 150 cc. phosphate buffer  $p_H$  5.0 and 25 g. of sodium nitrite dissolved in 175 cc. distilled water. The suspension was allowed to stand in the incubator for 1 hour after which it was centrifuged, the deposit of organism washed twice and suspended in 175 cc. saline.

The activations of the treated organism were determined, the results being noted in column *A* of Table XI. It will be seen that only feeble activations of lactic, formic, acetic and  $\alpha$ -hydroxybutyric acids remain and that for  $\alpha$ -glycerophosphoric acid has disappeared (*i.e.* its reduction time exceeds 3 hours). To 35 cc. of the nitrite-treated organism were then added a solution consisting of 25 cc. borate buffer solution  $p_H$  9.0 containing 2 g. sodium hydrosulphite and 2 cc. *N* NaOH solution. This suspension was allowed to stand in the incubator for 19 hours, after which it was centrifuged, twice washed and made up with normal saline to 35 cc. On determining the activations of the organism so treated it was found (see column *B*, Table XI, where all the results

<sup>1</sup> According to Böhm [1925] the particles in the well-known sol prepared by hydrolysing  $FeCl_3$  are mostly a basic iron chloride.



are noted) that the organism had regained some of its power to activate formic, acetic and  $\alpha$ -glycerophosphoric acids. The activity for lactic acid was somewhat diminished whilst that for the other donators had not re-appeared. When this partially restored organism was once more treated with nitrites the activities for all donators disappeared (column *C*, Table XI), and on re-treating with sodium hydrosulphite in the same way as before there was a slight restoration of the formic, acetic and  $\alpha$ -glycerophosphoric acid mechanisms. A control experiment (absence of hydrosulphite) showed no restoration whatever of the mechanisms eliminated by nitrites.

Table XI.

Conditions as in Table I. Concentration of substrates as in Table VIII.  
Reduction times.  $\infty$  = not reduced in 3 hours.

	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>
Succinic acid	$\infty$	$\infty$	$\infty$	—
Lactic "	50	72	$\infty$	Not reduced in 6 hrs.
Formic "	65	9	$\infty$	127
Acetic "	105	15	$\infty$	153
Butyric "	59	$\infty$	—	—
$\alpha$ -Hydroxybutyric acid	85	$\infty$	—	—
$\alpha$ -Glycerophosphoric acid	$\infty$	10	$\infty$	185
Glycerol	$\infty$	$\infty$	—	—
Glycol	$\infty$	$\infty$	—	—
Glutaminic acid	$\infty$	$\infty$	—	—
Glucose	$\infty$	$\infty$	—	—
Fructose	$\infty$	$\infty$	—	—
Mannose	$\infty$	$\infty$	—	—
Galactose	$\infty$	$\infty$	—	—
Mannitol	$\infty$	$\infty$	—	—
Sorbitol	$\infty$	$\infty$	—	—
Fumaric acid	0	0	—	—
Na nitrate	+	+	—	—
K chlorate	+	+	—	—

We have observed with another strain of *B. coli* the same restoring action of sodium hydrosulphite. 2 % cysteine, if used for a similar period at  $p_H$  7.4, appears to have a similar restoring action.

We do not think, however, that we have yet obtained the best conditions for maximum restoration. We have never observed any sign of restoration with an organism whose activating mechanisms have been entirely destroyed by heat.

### XIII. *Experiments of a preliminary nature on B. coli.*

A number of experiments of a preliminary (*i.e.* not of a systematic) nature have been carried out in connection with the reactions of *B. coli* and their results are summarised below.

1. If a suspension of *B. coli* be treated with copper sulphate solution for half an hour at a concentration of 1/5000 and the organism is well washed, the activations for glucose, succinic acid, lactic acid and formic acid are eliminated.

If now the treated organism be exposed to a current of  $H_2S$  for 4 minutes

and the organism (which appears black) be washed free from  $H_2S$  its subsequent reactions are as follows:

$$t_{\text{glucose}} = 45; t_{\text{succinic acid}} = \infty; t_{\text{lactic acid}} = 30; t_{\text{formic acid}} = 12.$$

The treatment with  $H_2S$  has resulted in the restoration of a number of the activating mechanisms.

A similar effect occurs on exposing *B. coli* to mercuric chloride solution (1/5000) for the same period. After treatment we obtain

$$t_{\text{glucose}} = \infty; t_{\text{succinic acid}} = \infty; t_{\text{lactic acid}} = 65; t_{\text{formic acid}} = 5.2.$$

On treating this organism with  $H_2S$  and after subsequent washing, the activations are

$$t_{\text{glucose}} = 28; t_{\text{succinic acid}} = \infty; t_{\text{lactic acid}} = 22; t_{\text{formic acid}} = 6.5.$$

The action of  $H_2S$  in restoring activity of a number of mechanisms inactivated by copper or mercury is similar to the restoring action of  $H_2S$  on invertase inactivated by silver [Myrbäck, 1926].

2. If to a suspension of *B. coli* glucose be added and the solution incubated at  $45^\circ$  for 2 hours, the reaction of the solution becomes distinctly acid, presumably due to the formation of lactic acid by glycolysis. Taking the fall of  $p_H$  as an indication of glycolysis we find that, whenever the organism is so treated that the activating power for glucose disappears, the glycolytic power of the organism also disappears. We have treated the organism in many different ways and have always observed this parallel disappearance of glycolytic power and glucose-activating power. The inference would appear to be that the two mechanisms—glycolysis and glucose activation—are controlled in *B. coli* by the same agency, or that a direct consequence of glucose activation by *B. coli* is glycolysis.

3. We have observed on several occasions that *B. coli* which has been so treated that its activity for glucose has disappeared is still capable of activating hexosediphosphate as a donator. There is, thus, a distinct difference between these two substances in relation to the activating power of *B. coli*.

We wish to emphasise, however, that the observations in this section are of a preliminary nature and have not yet been systematically investigated.

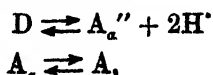
#### DISCUSSION.

In a previous paper [Quastel, 1926] the view was put forward that the activations of substrate molecules (hydrogen donators or acceptors) were due to polarisations of these molecules effected at locally intense electric fields whose presence and nature were dependent upon the characters of a surface structure. Such a polarisation implied the attachment (not necessarily permanent) of a substrate molecule to a particular grouping orientated in the surface, the field due to this grouping bringing about, provided that the strength of the field were sufficiently high, an activation of the substrate molecule.

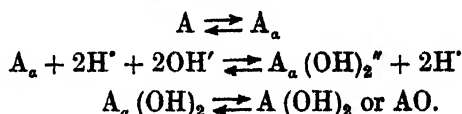
Evidence was given to show that the chief site of reduction of methylene

blue by substrates activated by *B. coli* was at the cell surface and further evidence was brought to show that, although activations of substrates may occur within the cell, such activations could not account for the entire reduction of methylene blue, and hence a site of activation of the substrate molecules was also at the cell surface. Upon the character of this surface as well as upon the chemical and electrical nature of the substrate molecules depended the variety of activations to which *B. coli* gives rise.

When activation of a substrate molecule (H donator) occurs, the following equations represent the events which follow:



where D is the hydrogen donator, A is the oxidised form after loss of 2H and  $A_a$  is the activated form of the molecule which can only exist as such in presence of the polarising field. The act of oxidation consists in the transference of electrons from  $A_a''$ , the electrically neutral molecule  $A_a$  being transformed into the normal form A out of contact with the field. The system thus becomes amenable to the same theoretical treatment as any system which gives rise to a reduction or oxidation potential. If A is a molecule whose oxidised form is AO, then, in presence of the ions of water, it may act as a H donator in the following way:



It follows from this that given a substrate activated by the cell to form a hydrogen donator, any hydrogen acceptor will react with it so long as the acceptor is already active (gives a reduction or oxidation potential), the equilibrium point being determined by the potentials of the two systems. Thus the cell (or enzyme) will evince no specificity to such acceptors. Similarly if a substrate is activated by the cell to form a hydrogen acceptor, this will react with H donators so long as the latter are already active (give reduction potentials) and no specificity will be evinced to such donators. Certain other considerations which will be described, are, however, to be taken into account when considering the question of specificity.

The conception of the mechanism of activation stated above does not imply that an electric field, at the surface, will activate *any* molecule which comes into contact with it. If such were the case, there could be none of the selectivity of destructive action, due to exposure to abnormal conditions, which actually occurs. What we actually expect, however, is that certain fields will be able to activate a series of molecules A, B, C ... X, whilst other fields, of weaker intensity, will only be able to activate the series, say, C ... X. This follows from the fact that the activation of a certain molecule necessitates the uptake of a critical amount of energy. Thus, if a surface structure possess

two fields *P* and *Q*, the first of which activates molecules *A*, *B* and *C* and the second (the less intense) only *C*, then if this structure be attacked in some way, the strength of *Q* may so diminish that it can no longer activate *C* whilst that of *P*, diminishing proportionately, may still retain sufficient power to activate *B* and *C* but not *A*. In this way selectivity of action by a surface structure may occur.

Apart, however, from this consideration a number of other factors playing a part in the phenomena of specificity must be taken into account. There is, for instance, the sign of the polarising field and, equally important, a set of factors which are perhaps best embraced by the term "accessibility." The most obvious of these factors is the stereochemical. If, for instance, a definite orientation of the substrate molecule at the surface structure is required for activation to occur, then the stereo-structure (and polarity) of the substrate molecule will be an all-important factor in determining such an orientation and hence an activation. Thus, if there are two groupings *P* and *Q* at a surface, both of which are capable, through the intensity and nature of their fields, of activating a molecule *A*, the character of the surface at *P* may be so different from that at *Q*, that the required orientation of *A* may occur at *P* but not at *Q*. In this way a sort of "lock and key" specificity is obtained. Again, one grouping *P* may be capable of activating two molecules *A* and *B*; yet the polarity and stereo-structure of the two molecules may be such as to favour the accessibility of *A* to this grouping and hence its activation but not that of *B*. Hardy and Doubleday's work [1921, 1922] on lubrication serves to indicate the importance of polarity in a molecule in determining its orientation at a surface or its accessibility to a structure.

There is another factor of some importance. If in presence of a *H* donator, activated by an enzyme, there is present either of two acceptors both of which give the same reduction potentials, the final equilibrium point (assuming reversibility) will be the same in both cases. The rate at which this point is achieved by the two systems may, however, be greatly different.

This is indicated in Table XII where it is shown that the velocity of reduction of different dyestuffs is greatly different from what would be anticipated if the velocity of reaction were proportional to the difference in reduction potential between the reacting substances. This has also been shown by Dixon [1926] in the case of xanthine oxidase. It is easy to see why this is so, for the slow reducing indicators are the acid dyestuffs which are not so accessible to, or not so easily adsorbed by, the negatively charged cell as the more basic compounds. If the reduction occurs chiefly at the cell surface, then since the velocity of reduction is governed by the concentration of indicator as well as by the concentration of activated donator at the surface, it follows that "accessibility" of the indicator is a determining factor in controlling the velocity. Similarly, the same type of "accessibility" of a substrate molecule which has to be activated may be a determining factor in controlling the velocity of reaction. In certain cases the velocity of the latter may be so small

as to give the appearance of inactivity and we should conclude that an enzyme capable of activating the substrate in question was absent. This would be a legitimate conclusion so long as it were understood that the sole criterion for specificity of action is the velocity of reaction.

Table XII.

*Coli* 13. Conditions as in Table I, but with different dyestuffs as H acceptors.  
 $\infty$  = not reduced within 4 hours.

Dyestuff <i>M</i> /1880	<i>t</i> <sub>formic acid</sub>	<i>t</i> <sub>lactic acid</sub>	<i>t</i> <sub>succinic acid</sub>	<i>t</i> <sub>glucose</sub>	Approx <i>r</i> <sub>H</sub> range
Indigo carmine	$\infty$	$\infty$	$\infty$	$\infty$	7.2-11.2
K indigotrisulphonate	$\infty$	$\infty$	$\infty$	$\infty$	8.3-12.3
K indigotintetrasulphonate	$\infty$	$\infty$	$\infty$	$\infty$	10 -14
Methylene blue	4.7	7.5	9.5	5.5	13 -17
1-Naphthol-2-sulphonate-2 : 6-dichlorophenol	180	180	$\infty$	150	15.2-19.2
1-Naphthol-2-sulphonate-indophenol	130	118	183	125	15.4-19.4
<i>o</i> -Cresol-2 : 6-dichloroindophenol	45.5	42.5	55.5	43	17.3-21.3
<i>o</i> -Cresol-indophenol	19.5	13.1	13.2	11.5	17.7-21.7
2 : 6-Dibromophenol-indophenol	36.2	32	45	41	18.3-22.3
<i>o</i> -Chlorophenol-indophenol	16.5	13	13.7	14	19 -23

The results given in this paper show very clearly how certain activating mechanisms can be regarded as more resistant, others less resistant to exposure to abnormal conditions. As the time of exposure increases, there is a gradual, "step by step" disappearance of all the mechanisms. The order of sensitivity or disappearance is, on the whole, the same. Even in the case of the apparent restoration of some of these mechanisms (Section XII) it is those mechanisms which are last to disappear which return.

The sugars form an interesting class in that they appear to be associated with a particular portion or patch of the surface. This is indicated by results given in Sections VII to X. We consider that the best interpretation of the results is afforded by the assumption that on exposure of *B. coli* to ether, chloroform, benzene, toluene, phenol, etc., a film of the substance is formed upon certain patches of the cell surface, these portions being particularly associated with the activations of the sugars, and possibly being of a fatty or lipoidal nature. The formation of such a film would remove or diminish the accessibility of such highly polar compounds as the sugars and polyhydric alcohols and hence have the effect of eliminating the activating mechanisms of the latter. It is possible, too, that the actual solution of such substances as ether, etc., in the more fatty or lipoidal fractions of the surface will so alter the surface structure as entirely to destroy the groupings associated with the activations of the sugars.

It may be urged that the effect of ether, toluene, etc., is to dissolve off from the organism some material essential for the activation of the sugars, etc. This seems unlikely when it is considered

(1) that a small quantity of ether (benzene or toluene) or a short exposure is just as effective in removing all action with the sugars as a prolonged extraction with ether;

- (2) that 1 % phenol has exactly the same effects as ether;
- (3) that an activation for fructose appears to persist even after thorough extraction with ether.

Again, the action of ether, etc., cannot be interpreted as due to the alteration of cell permeability (though this doubtless occurs) when it is considered that although the treated cell is now presumably impermeable to glycol, glycerol and the sugars it must be still permeable to such relatively large molecules as methylene blue and  $\alpha$ -glycerophosphoric acid.

The activations of succinic acid, formic acid, etc., appear to be associated with the less fatty or lipoidal fractions of the surface. This is indicated in Sections V and VII-X.

The experiment with "dialysed iron" (Section XI) indicates that a loose union with the organism, such as might be expected when oppositely charged particles mutually precipitate, has but little effect on the activations of the organism.

It is of interest that the mechanisms dealing with succinic acid and fumaric acid appear to be identical (see Tables VIII, IX, X). In every case when the succinic acid activation disappears so does that for fumaric acid. No selective action on the two mechanisms has been observed.

Summing up, it seems to us that the experimental results favour the views which have been put forward. No other interpretation appears satisfactorily to fit all the facts.

It may, perhaps, be emphasised once more that on these views a surface structure may induce a variety of reactions each differing in some way from the other so that the appearance of a large number of specific enzymes is obtained. The cell surface—or an intracellular surface—can be regarded as an assemblage of such enzymes but "enzymes which are inseparable because they are actually a function of the surface structure." As such structures become smaller in size the variety of reactions to which they give rise will become smaller and hence the specificity of its effects will apparently become greater until eventually a structure may be reached for which high specificity may be claimed. But there is no fundamental difference between such a highly specific structure and that of a cell surface (such as *B. coli*) which can accomplish over 50 activations.

#### SUMMARY.

1. Resting *B. coli* was exposed to the following conditions and substances, and its activations, after exposure, were determined by the methylene blue technique.

- (a) Changes of temperature from that of liquid air to 77°.
- (b) Changes of H ion concentration from  $p_H$  2.2 to  $p_H$  11.
- (c) Varying concentrations of sodium chloride at varying  $p_H$  and for different times.

- (d) Varying concentrations of sodium nitrite at varying  $p_H$  and for different times.
- (e) Methylene blue.
- (f) Benzene, toluene, phenol, ether, chloroform, propyl alcohol and *cyclo*-hexanol.
- (g) "Dialysed iron."
- (h) Dilute solutions of  $\text{CuSO}_4$  and  $\text{HgCl}_2$  (preliminary experiments only).

2. Selectivity of destructive action on the activating mechanisms is exhibited by such exposures. A step by step degradation occurs. The order of sensitivity or of disappearance of the mechanisms is approximately the same in all cases.

3. The activations of the sugars appear to take place at the more fatty or lipoidal fractions of the surface; those of succinic acid, formic acid, etc., at the less lipoidal. It would seem that the activation of glucose as a H donator leads also to glycolysis.

4. Reversibility of action is demonstrated in the cases of propyl alcohol and *cyclo*-hexanol, and in the restoring effects of  $\text{H}_2\text{S}$  on mechanisms inactivated by copper or mercury. A restorative action by sodium hydrosulphite is described.

5. The experimental results are in favour of the view, previously propounded, that the activations of substrates are intimately associated with surface structure. The specificity of behaviour observed, as deduced from the selectivity of destructive action, is shown to be in agreement with this view of surface activation.

It is a great pleasure to express our sense of gratitude to and our appreciation of the interest taken in this work by Sir F. G. Hopkins. One of us (W. R. W.) is indebted to the Medical Research Council for a part time grant.

#### REFERENCES.

- Böhm (1925). *Z. anorg. Chem.* **149**, 203.  
Cole and Onslow (1916). *Lancet*, ii, 9.  
Dixon (1926). *Biochem. J.* **20**, 714.  
Hardy and Doubleday (1921). *Proc. Roy. Soc. Lond. A.* **100**, 550.  
—— (1922). *Proc. Roy. Soc. Lond. A.* **101**, 487.  
Myrbäck (1926). *Z. physiol. Chem.* **158**, 160.  
Nicolai (1926). *Biochem. Z.* **179**, 86.  
Quastel (1926). *Biochem. J.* **20**, 166.  
Quastel and Whetham (1925, 1). *Biochem. J.* **19**, 520.  
—— (1925, 2). *Biochem. J.* **19**, 645.  
Quastel and Wooldridge (1925). *Biochem. J.* **19**, 652.

## XXIII. CHEMISTRY OF THYROXINE.

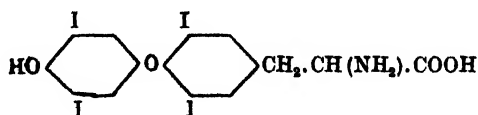
### III. CONSTITUTION AND SYNTHESIS OF THYROXINE.

By CHARLES ROBERT HARINGTON AND GEORGE BARGER.

*From the Department of Pathological Chemistry, University College Hospital Medical School, London, and the Department of Medical Chemistry, University of Edinburgh.*

(Received December 29th, 1926.)

In recently published work it has been shown by one of us [Harington, 1926] that thyroxine is a tetraiodo-derivative of the *p*-hydroxyphenyl ether of tyrosine<sup>1</sup>; the orientation of the iodine atoms was left undetermined but it was suggested that they probably occupied the positions shown in the following formula:



The object of the present paper is in the first place to offer evidence in support of this suggestion, and in the second place to describe the synthesis of thyroxine itself<sup>2</sup>.

The experimental and theoretical considerations leading to the above-mentioned supposition regarding the position of the iodine atoms were as follows.

(1) Thyroxine was fused with potash at a high temperature in the absence of oxygen, in the hope of obtaining identifiable degradation products in which iodine was replaced by hydroxyl. Although no pure substances could be isolated as the result of these experiments, the products of alkali fusion did, nevertheless, exhibit pyrogallol reactions, from which it appeared probable that one or both of the benzene rings of the thyroxine molecule had been converted into 3:4:5-trihydroxy-derivatives.

(2) Kendall and Osterberg [1919] have described a colour reaction with nitrous acid and ammonia as being characteristic of thyroxine; we have found that this colour reaction is given in general by benzene derivatives which contain two iodine atoms in the *ortho* positions to a hydroxyl (or amino-)

<sup>1</sup> I have since learned that Dr H. D. Dakin had come simultaneously to substantially the same conclusions as myself regarding the constitution of thyroxine; he had made the additional interesting observation that, on heating thyroxine with fuming hydriodic acid at 140°, tyrosine is formed. On hearing from Prof. Barger that I had communicated a paper on the subject to this *Journal*, Dr Dakin withdrew his paper, which was at that time in the hands of the Editor of the *Journal of Biological Chemistry*, from publication. C. R. H.

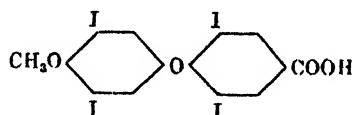
<sup>2</sup> The claim of Kendall and Osterberg [1919] to have synthesised thyroxine cannot be maintained in view of the facts that no experimental evidence has been offered in support of this claim, and, further, that the views of these authors regarding the constitution of thyroxine have been shown [Harington, 1926] to be wholly erroneous.



group; it is, for instance, given with intensity by diiodo-*p*-cresol, by 3:5-diiodo-4-hydroxybenzoic acid, and by 3:5-diiodotyrosine; this therefore made it likely that thyroxine contained a similar grouping.

(3) On general grounds one is almost bound to regard thyroxine as being derived from tyrosine, through the stage of 3:5-diiodotyrosine; assuming this to be the case, it is highly probable that thyroxine is formed in nature by the coupling of two molecules of diiodotyrosine with the loss of one side chain; such a reaction would lead to a compound of the suggested constitution.

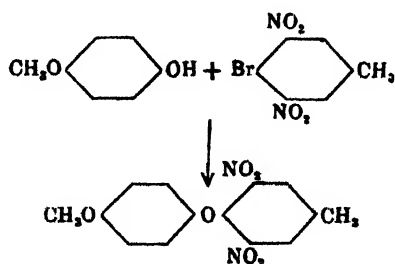
Admittedly these arguments cannot be regarded as convincing, but further evidence by methods of degradation appeared to be out of the question, and the solution of the problem could therefore only be obtained by synthesis. A certain simplification could indeed be effected by the methylation and subsequent oxidation of thyroxine, by a series of steps precisely similar to that described in the case of desiodothyroxine [Harington, 1926] leading to a tetraiodo-derivative of the acid  $\text{CH}_3\text{O} \cdot \text{C}_6\text{H}_4 \cdot \text{O} \cdot \text{C}_6\text{H}_4 \cdot \text{COOH}$ , and our first object was therefore the synthesis of the compound



and its comparison with the corresponding acid derived from thyroxine.

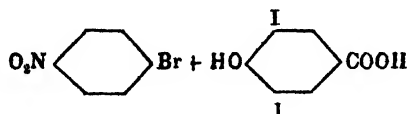
Early experiments on the direct iodination of desiodothyroxine itself and of other *p*-hydroxyphenyl ethers had indicated that these compounds would take up readily two atoms of iodine (though the products were very difficult to purify), but that the uptake of iodine ceased definitely at this point; no method was found for the direct introduction of more than two atoms of iodine. This being so, it was apparent that the desired tetra-iodophenyl ethers could only be obtained by the introduction of those two iodine atoms (or other groups which could be substituted by iodine) which were destined to occupy the 3:5 positions, *before* the phenyl ether synthesis was carried out. In other words, iodine atoms (or other replaceable groups) had to be present in the *ortho* positions either to the halogen atom or to the phenolic group which was to take part in the phenyl ether condensation.

In the first experiments in this direction quinol monomethyl ether was condensed with 3:5-dinitro-4-bromotoluene:



the resulting dinitrodiphenyl ether was successfully reduced to the diamine, but complete failure attended all attempts to replace the amino-groups in this compound by iodine.

This line of attack was therefore abandoned in favour of attempts to condense *p*-nitrohalogenbenzenes with 3:5-diiodo-4-hydroxybenzene derivatives, *e.g.* to condense *p*-nitrobromobenzene with 3:5-diiodo-4-hydroxybenzoic acid:

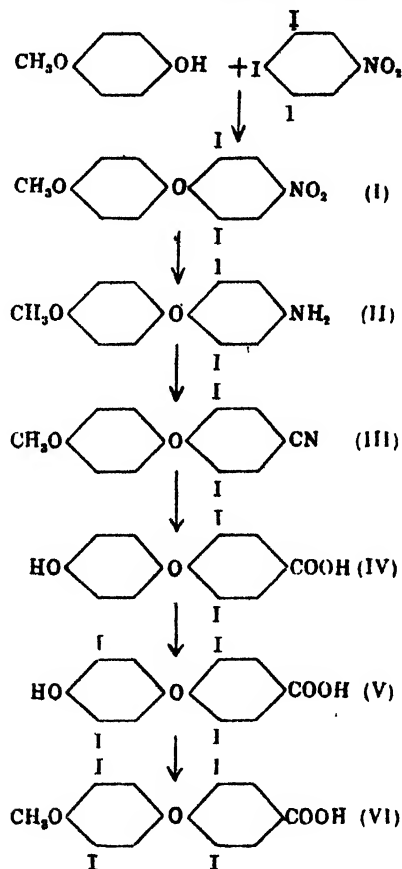


Much time was spent without success on experiments of this type. Mild treatment, such as warming the components in pyridine solution, or boiling in acetone with potassium carbonate, resulted only in the recovery of unchanged starting material, whilst more vigorous treatment, with the addition of copper bronze, caused the elimination of the relatively labile iodine atoms.

The solution of the difficulty was finally obtained in a different manner. As is well known, the presence of a nitro-group exerts a powerful mobilising effect on a halogen in the *para* position, a somewhat less marked effect on one in the *ortho* position, and no effect at all on one in the *meta* position. In accordance with this fact it was found that, of the three iodine atoms in 3:4:5-triiodonitrobenzene, that in the 4-position is so far preferentially mobilised that this substance can be condensed with a phenol to give a good yield of the 3:5-diiodo-4-phenoxy-nitrobenzene. Quinol monomethyl ether, therefore, was condensed with 3:4:5-triiodonitrobenzene to give 3:5-diiodo-4-(4'-methoxyphenoxy)nitrobenzene (I); this compound was reduced to the corresponding aniline (II) and the latter converted, by means of Sandmeyer's reaction, into the nitrile (III). This nitrile, on boiling with a mixture of hydriodic and acetic acids, underwent simultaneous hydrolysis and demethylation, yielding 3:5-diiodo-4-(4'-hydroxyphenoxy)benzoic acid (IV); on addition of iodine in potassium iodide to a solution of this acid in concentrated ammonia, iodine was rapidly taken up, the uptake ceasing abruptly at two molecules, and there was obtained a good yield of 3:5-diiodo-4'-(3':5'-diiodo-4'-hydroxyphenoxy)benzoic acid (V); on methylation, this gave an acid (VI) which was in every respect identical with the acid obtained by the methylation and subsequent oxidation of thyroxine. This series of experiments, therefore, settled beyond doubt the question of the orientation of the iodine atoms in thyroxine, since it is certain that the last two iodine atoms, introduced into the acid (IV) in alkaline solution, must have entered the *ortho* positions to the free phenolic group.

As regards the actual synthesis of thyroxine, the most favourable starting point appeared to be 3:5-diiodo-4-(4'-methoxyphenoxy)benzaldehyde (VII) which could indeed be obtained in good yield from the nitrile (III) by the method of Stephen [1925]. It was obvious, however, that formidable diffi-

culties were to be expected, since the reduction which would be involved in the synthesis, from this aldehyde, of the corresponding  $\alpha$ -aminopropionic acid would be likely to displace the iodine atoms; indeed, for this reason, an alkaline reduction was entirely out of the question, and prolonged heating with hydriodic acid and red phosphorus proved to be equally unsuccessful. The first success was obtained by condensing the aldehyde with hydantoin, boiling the product for one hour with hydriodic acid and a little phosphorus,



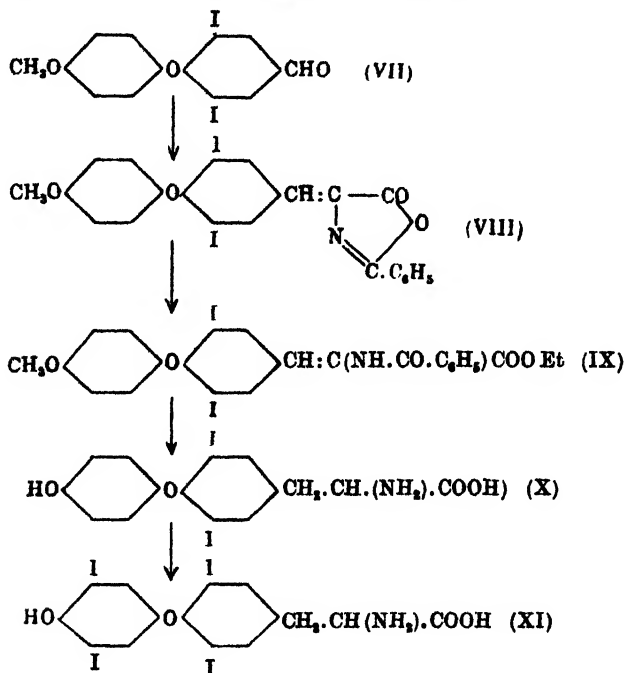
and hydrolysing the resulting compound with concentrated barium hydroxide; the intermediate products were, however, very insoluble and difficult to purify, and the yields were poor. Considerably better results were obtained as follows: the aldehyde (VII) was condensed with hippuric acid, and the resulting azlactone (VIII) converted into the corresponding  $\alpha$ -benzoylamino-cinnamic ester (IX); this, on boiling for one hour with hydriodic acid and red phosphorus, gave a 25 % yield of

$\beta$ -[3:5-diiodo-4-(4'-hydroxyphenoxy)]phenyl- $\alpha$ -aminopropionic acid (X).

On iodination in ammoniacal solution this compound yielded

$\beta$ -[3:5-diiodo-4-(3':5'-diiodo-4'-hydroxyphenoxy)]phenyl- $\alpha$ -aminopropionic acid (XI),

a substance which was entirely identical, in its chemical and physiological properties, with thyroxine isolated from the thyroid gland. By this synthesis, therefore, together with the confirmatory evidence offered by the synthesis of the iodine-containing degradation product described above, the constitution of thyroxine may be regarded as finally established.



# EXPERIMENTAL.

## A. Degradation of thyroxine.

**Potash fusion of thyroxine.** 0.5 g. thyroxine was mixed with 5 g. powdered potassium hydroxide and the mixture heated at  $310^\circ$  for 30 minutes in a silver tube through which a current of hydrogen was passing. After cooling, the melt was dissolved out with hydrochloric acid containing some sulphur dioxide. The product was not entirely soluble, a grey precipitate being obtained, which, as also the solution itself, darkened on exposure to air. On addition of excess of alkali the solution became immediately black. When the solution was carefully neutralised with ammonia and treated with dilute ferric chloride, colour reactions of the pyrogallol type were obtained. Variations of the duration and temperature of the fusion were tried, but in no case could any pure product be isolated.

**Exhaustive methylation of thyroxine.** 2.33 g. thyroxine were dissolved in 55 cc. methyl alcohol containing 1 % potassium hydroxide; 25 cc. of methyl iodide were added and the solution boiled under a reflux condenser; boiling was continued for 8 hours in all, additions of 10 cc. of 4 % methyl alcoholic

potassium hydroxide being made at half-hourly intervals, and two further quantities of 5 cc. of methyl iodide being added at 4 and 6 hours respectively; 100 cc. of water were then added and the methyl alcohol and excess of methyl iodide distilled off under reduced pressure; the colourless product which separated was filtered off and boiled with 50 cc. alcohol and 50 cc. 2*N* potassium hydroxide until trimethylamine ceased to be evolved; the hot clear solution, on cooling, deposited 1.6 g. of the potassium salt of the unsaturated acid. The free acid, on crystallisation from glacial acetic acid, in which it was but little soluble, formed fine colourless needles which gave off iodine at 286° and melted indefinitely above 290°.

*Analysis.* 5.747 mg. gave 5.340 mg. CO<sub>2</sub>; 0.80 mg. H<sub>2</sub>O.  
1.272 mg. required 7.73 cc. *N*/196 thiosulphate [Kendall, 1914].

	C	H	I
Found	25.3 %	1.5 %	65.75 %
Calc. for C <sub>16</sub> H <sub>10</sub> O <sub>4</sub> I <sub>4</sub>	24.8 %	1.3 %	65.5 %

*Oxidation of unsaturated acid, C<sub>16</sub>H<sub>10</sub>O<sub>4</sub>I<sub>4</sub>.* 0.81 g. of the potassium salt of the above acid was suspended in 35 cc. boiling water, and treated gradually with 1.5 *N* potassium permanganate; after the latter had been added in amount corresponding to 3 atoms of oxygen the oxidation ceased abruptly; apparently the aldehyde is so completely insoluble in water as to escape further oxidation under these conditions. The solution was treated with sulphur dioxide, and the amorphous precipitate, consisting entirely of neutral material, was filtered off, washed, dried and crystallised from glacial acetic acid; it formed sheaves and rosettes of fine colourless needles, M.P. 198°.

*Analysis.* 4.515 mg. gave 3.890 mg. CO<sub>2</sub>; 0.51 mg. H<sub>2</sub>O.  
1.37 mg. gave 0.9489 mg. I [Kendall, 1914].

	C	H	I
Found	23.5 %	1.3 %	69.3 %
Calc. for C <sub>14</sub> H <sub>8</sub> O <sub>3</sub> I <sub>4</sub>	23.0 %	1.1 %	69.5 %

*Oxidation of aldehyde, C<sub>14</sub>H<sub>8</sub>O<sub>3</sub>I<sub>4</sub>.* 0.2 g. of the above aldehyde was dissolved in 4 cc. pure pyridine and the solution treated gradually, in the cold, with 0.6 cc. of 5 % potassium permanganate; after the latter had been entirely decolorised, the solution was diluted with water, treated with sulphur dioxide until colourless, made acid to Congo red with hydrochloric acid, and the white precipitate filtered off. The product was entirely soluble in a large amount of boiling dilute sodium carbonate, but, on cooling, the sodium salt separated almost completely in well-formed colourless needles. The free acid, crystallised from glacial acetic acid, formed a felt of fine colourless needles, M.P. 283°.

*Analysis.* 4.443 mg. gave 3.720 mg. CO<sub>2</sub>; 0.53 mg. H<sub>2</sub>O.  
1.466 mg. gave 1.0019 mg. I [Kendall, 1914].

	C	H	I
Found	22.8 %	1.3 %	68.3 %
Calc. for C <sub>14</sub> H <sub>8</sub> O <sub>4</sub> I <sub>4</sub>	22.5 %	1.1 %	67.9 %

The *methyl ester*, prepared in the usual way, and recrystallised from acetic acid, formed flattened rhombic prisms, M.P. 233°; the *ethyl ester* formed long thin needles, M.P. 171.5°.

B. *Synthesis of acid degradation product, C<sub>14</sub>H<sub>9</sub>O<sub>4</sub>I<sub>4</sub> (VI).*

3:4:5-Triiodonitrobenzene was most conveniently prepared from diiodo-*p*-nitraniline (obtained by the method of Willgerodt and Arnold [1901]) as follows: 75 g. diiodo-*p*-nitraniline were dissolved in 150 cc. concentrated sulphuric acid; the solution was cooled to 5° and vigorously stirred while finely powdered sodium nitrite (2.5 mols.) was slowly added; the stirring was continued for a further 2 hours, the temperature being maintained at 5–10° throughout. The mixture was then poured on to ice and treated with a concentrated aqueous solution of potassium iodide (5 mols.). After the first vigorous reaction, the solution was warmed for some time on the water-bath; it was then cooled, free iodine was removed with bisulphite, and the granular precipitate filtered off, washed with water, alcohol and ether, and dried. The most convenient purification was effected by distillation *in vacuo*, which gave a clean product, M.P. 165°, in a yield of 75–80 % of the theoretical. The M.P. of this compound is given by Willgerodt and Arnold [1901] as 105°; in a later paper by Repossi [1916] the figure given is 167°<sup>1</sup>.

3:5-Diiodo-4-(4'-methoxyphenoxy)nitrobenzene (I). This compound was prepared by boiling a solution of quinol monomethyl ether and 3:4:5-triiodonitrobenzene in methylethylketone with dry potassium carbonate. If molecular proportions were employed, the product contained a good deal of unchanged triiodonitrobenzene which is difficult to remove; it was found better, therefore, to use twice the theoretical amount of the phenol and potassium carbonate. It is further essential for the success of this reaction that the potassium carbonate should be freshly dried and finely powdered. 50 g. 3:4:5-triiodonitrobenzene and 25 g. quinol monomethyl ether were dissolved in 250 cc. boiling methylethylketone; 30 g. finely powdered anhydrous potassium carbonate were added and the solution boiled under a reflux condenser for 16 hours; it was then poured into water containing a slight excess of acetic acid, and the methylethylketone and excess of quinol monomethyl ether were removed by steam distillation; after cooling, the precipitate was filtered off, and crystallised, first from glacial acetic acid, and then from methylethylketone; there resulted 33–35 g. of a product which was practically pure, *i.e.* a yield of about 67 %. For complete purification the compound was distilled *in vacuo* (B.P. 260°/3 mm.) and crystallised from methylethylketone. It formed yellow elongated prisms, M.P. 144°. It is insoluble in water, slightly soluble in alcohol and ether, much more readily so in chloroform and acetic acid.

*Analysis.* 0.2257 g. gave 0.2593 g. CO<sub>2</sub>; 0.0474 g. H<sub>2</sub>O.  
0.1581 g. gave 0.1502 g. AgI.  
20.1 mg. gave 0.523 mg. N (modified micro-Kjeldahl).

	C	H	N	I
Found	31.3 %	2.3 %	2.6 %	51.3 %
Calc. for C <sub>18</sub> H <sub>9</sub> O <sub>4</sub> NI <sub>4</sub>	31.2 %	1.8 %	2.8 %	51.1 %

<sup>1</sup> This compound has also been recently prepared in a similar manner by Kalb, Schmeizer, Zellner and Berthold (*Ber. deutsch. chem. Ges.* 1926, 59, 1860. [C. H. R. February 2, 1927.]

3:5-Diiodo-4-(4'-methoxyphenoxy)aniline (II). 25 g. of the above nitro-compound were dissolved in 125 cc. hot glacial acetic acid; 37.5 g. powdered stannous chloride were added, and a rapid stream of dry hydrogen chloride passed into the solution, which was kept hot on the water-bath. After a time the stannichloride of the base began to separate; when the precipitate no longer increased in amount and the solution was saturated with hydrochloric acid (about 45 minutes), it was allowed to cool; the crystalline stannichloride was then filtered off, washed with glacial acetic acid, decomposed as rapidly as possible by grinding with 250 cc. of 40 % sodium hydroxide, and the base immediately extracted with ether. If the operation had been properly conducted, the ethereal solution was but little coloured, in which case it was used, after drying, for the direct preparation of the hydrochloride of the base which was required for the next reaction. The free base was obtained pure by evaporating the dried ethereal solution and recrystallising the residue, first from a very small amount of benzene, and then from ligroin. It formed colourless narrow rhombic prisms, M.P. 121–122°. It was very slightly soluble in hot water, but easily soluble in most organic solvents except light petroleum.

Analysis. 0.1782 g. gave 0.2204 g.  $\text{CO}_2$ ; 0.0426 g.  $\text{H}_2\text{O}$ .  
 0.1629 g. gave 0.1634 g. AgI.  
 34.2 mg. gave 1.024 mg. N (micro-Kjeldahl).

	C	H	N	I
Found	33.7 %	2.6 %	2.7 %	54.2 %
Calc. for $\text{C}_{15}\text{H}_{11}\text{O}_2\text{NI}_2$	33.4 %	2.4 %	3.0 %	54.4 %

The *hydrochloride* formed colourless needles, sparingly soluble in water, M.P. 216° after preliminary sintering; the *sulphate* formed colourless needles, with similar solubilities, M.P. 201° after sintering. The base and its salts have a distinctly irritating effect on the skin, which necessitates considerable care in handling them.

3:5-Diiodo-4-(4'-methoxyphenoxy)benzonitrile (III). The diazotisation of the base just described offered, at first, considerable difficulties, since, in the presence of excess of mineral acid (especially of sulphuric acid) complex coloured condensation products were readily formed. The reaction can however be conveniently accomplished by treatment of a suspension of the dry hydrochloride in glacial acetic acid with amyl nitrite. 30 g. of the hydrochloride, prepared by saturating the ethereal solution of the base with dry hydrogen chloride, were suspended in 300 cc. of glacial acetic acid; the suspension was vigorously stirred and kept at 15–20° while amyl nitrite was gradually added in slight excess; the hydrochloride rapidly disappeared and there was obtained a clear orange-coloured solution of the diazonium salt. For the preparation of the nitrile, this solution was poured, with stirring, into a hot solution prepared by adding 170 g. potassium cyanide in 300 cc. water to 150 g. copper sulphate in 600 cc. water. (It was found to be necessary to use the very large excess of cuprous cyanide here indicated, since otherwise the diazonium group was very readily exchanged for hydrogen.) The precipitate was filtered off, washed with water, and boiled out with benzene; the

benzene solution was filtered and dried over calcium chloride. The benzene was then evaporated and the residue distilled under reduced pressure; the dark-coloured, but crystalline, distillate was recrystallised from methylethylketone, and 13–15 g. of a substance forming colourless prisms, m.p. 167–169°, were obtained.

*Analysis.* 0.2145 g. gave 0.2760 g.  $\text{CO}_2$ ; 0.0382 g.  $\text{H}_2\text{O}$ .  
0.1650 g. gave 0.1625 g. AgI.  
20.0 mg. gave 0.574 mg. N (micro-Kjeldahl).

	C	H	N	I
Found	35.1 %	2.0 %	2.9 %	53.2 %
Calc. for $\text{C}_{14}\text{H}_9\text{O}_2\text{NI}_2$	35.2 %	1.9 %	2.9 %	53.2 %

3:5-Diiodo-4-(4'-hydroxyphenoxy)benzoic acid (IV). 5 g. of the nitrile were dissolved in 40 cc. glacial acetic acid, and to the boiling solution, contained in a flask under a reflux condenser, were slowly added 50 cc. hydriodic acid (Sp. G. 1.7) so as to cause as little precipitation of the nitrile as possible; the whole was then boiled for 2 hours. On cooling and diluting with water a partly crystalline precipitate separated, which was filtered off and dissolved in warm dilute ammonia; the filtered ammoniacal solution was acidified and the acid filtered off, dried and crystallised from 50 % alcohol; it formed long colourless needles, m.p. 252–254°. The yield was 80 % of the theoretical.

*Analysis.* 0.1683 g. gave 0.2004 g.  $\text{CO}_2$ ; 0.0275 g.  $\text{H}_2\text{O}$ .  
0.1626 g. gave 0.1592 g. AgI.

	C	H	I
Found	32.5 %	1.8 %	52.8 %
Calc. for $\text{C}_{12}\text{H}_6\text{O}_4\text{I}_2$	32.3 %	1.7 %	52.7 %

During the above-described hydrolysis there was always formed a certain amount of a compound containing no nitrogen, and exhibiting phenolic but no acidic properties; this was not investigated further.

3:5-Diiodo-4-(3':5'-diiodo-4'-hydroxyphenoxy)benzoic acid (V). Attempts to iodinate the diiodo-acid, by the usual method for phenols, in solution in sodium or potassium hydroxide, met with no good result; the reaction proceeded smoothly, however, in ammoniacal solution by the method of Datta and Prosad [1917]. 0.964 g. of the diiodo-acid was dissolved in 8 cc. of concentrated ammonium hydroxide (Sp. G. 0.880); to this solution was added, drop by drop, with constant shaking, a normal solution of iodine in potassium iodide; as each drop was added the black precipitate of nitrogen iodide disappeared instantaneously, on shaking, until almost the theoretical 2 mols. of iodine had been added; with further addition of iodine, the black precipitate disappeared more slowly, until, when the theoretical amount (or one or two drops in excess) had been added, the dark colour became permanent, indicating the presence of nitrogen iodide in excess; the end-point of the reaction was almost as sharply defined as that of a titration. After about 75 % of the iodine had been added a crystalline ammonium salt began to separate and increased in amount until the end of the reaction; addition of saturated ammonium chloride solution precipitated still more of this salt, the total amount of which was 93 % of the theoretical for the ammonium salt of the



tetra-iodo-acid. The free acid, crystallised from glacial acetic acid, formed bunches of colourless needles, M.P. 255°, with decomposition and evolution of iodine.

Analysis. 0.1045 g. gave 0.1325 g. AgI.  
 Found I 68.5 %      Calc. for  $C_{12}H_5O_4I_4$  I 69.2 %

3:5-Diiodo-4-(3':5'-diiodo-4'-methoxyphenoxy)benzoic acid (VI). On methylation with dimethyl sulphate and potassium hydroxide in the usual manner, the above-described tetra-iodohydroxy-acid yielded the methoxy-acid, which, when crystallised from glacial acetic acid, formed a felt of colourless needles, M.P. 286°.

Analysis. 1.31 mg. required 8.4 cc. N/200 sodium thiosulphate [Kendall, 1914].  
 Found I 68.0 %      Calc. for  $C_{14}H_5O_4I_4$  I 67.9 %

The sodium salt of the acid was very sparingly soluble in water and crystallised in fine needles; the methyl ester, crystallised from acetic acid, formed flattened rhombic prisms, M.P. 233.5°; the ethyl ester similarly gave long fine needles, M.P. 172.5°. The results of comparison of this acid and its derivatives with the corresponding compounds obtained, as described above, by degradation of thyroxine are summarised in Table I; they leave no doubt as to the identity of the synthetic acid with that obtained from natural sources.

Table I.

	Crystalline form	M.P.	Mixed M.P.
Synthetic acid	Felted needles	286°	283-4°
Natural acid	"	283°	
Synthetic methyl ester	Flattened rhombic prisms	233.5°	233°
Natural "	"	233°	
Synthetic ethyl ester	Long needles	172.5°	172.5°
Natural "	"	171.5°	

### C. Synthesis of thyroxine.

3:5-Diiodo-4-(4'-methoxyphenoxy)benzaldehyde (VII). Our first attempts to prepare this aldehyde from the corresponding nitrile, using the conditions recommended by Stephen [1925], i.e. 1.5 mols. of stannous chloride and a period of 2 hours' standing, resulted in minute yields only. Much better results were obtained by increasing the proportion of stannous chloride to 4 mols., and allowing the mixture to stand overnight, or even longer. 5 g. of 3:5-diiodo-4-(4'-methoxyphenoxy)benzonitrile (see above) were dissolved in 35 cc. chloroform and added, with vigorous shaking, to a solution of 12 g. anhydrous stannous chloride in 60 cc. dry ether saturated with hydrogen chloride; after standing for at least 16 hours the yellow precipitate was filtered off, washed with ether and warmed with water containing a little hydrochloric acid; at the boiling point the hydrolysis was almost instantaneous, the yellow stannichloride being replaced by a colourless flocculent precipitate of aldehyde; this was filtered off, washed with dilute hydrochloric acid and then with water, dried, and crystallised from glacial acetic acid; it formed colourless prisms, M.P. 121°. The yield varied from 70 % to 100 % of the theoretical. The

aldehyde was practically insoluble in water, but fairly soluble in all organic solvents except light petroleum.

*Analysis.* 0.1643 g. gave 0.2131 g.  $\text{CO}_2$ ; 0.0324 g.  $\text{H}_2\text{O}$ .  
0.1479 g. gave 0.1447 g.  $\text{AgI}$ .

	O	H	I
Found	35.3 %	2.2 %	52.9 %
Calc. for $\text{C}_{14}\text{H}_{10}\text{O}_2\text{I}_2$	35.0 %	2.1 %	52.9 %

The *phenylhydrazone* formed long yellow needles, M.P. 175–176°.

*Aslactone from above aldehyde and hippuric acid* (VIII). An intimate mixture of 5 g. of the aldehyde, 5 g. freshly fused sodium acetate, and 1.9 g. hippuric acid was treated with 15 cc. acetic anhydride and heated on the water-bath for 15 minutes; the yellow reaction mixture was ground up with water, filtered off, well washed and dried; the yield was 6 g. of an almost pure product. Crystallised from glacial acetic acid, it formed long, bright yellow needles, M.P. 211°.

*Analysis.* 15.5 mg. gave 0.303 mg. N (micro-Kjeldahl).  
0.1539 g. gave 0.1174 g.  $\text{AgI}$ .

	N	I
Found	2.0 %	41.2 %
Calc. for $\text{C}_{22}\text{H}_{16}\text{O}_4\text{NI}_2$	2.2 %	40.9 %

*$\alpha$ -Benzoylamino-3:5-diiodo-4-(4'-methoxyphenoxy)cinnamic ester* (IX). The aslactone was warmed on the water-bath for 45–60 minutes with 75 parts of absolute alcohol containing 10 % sulphuric acid; the clear and almost colourless solution was poured into water and the precipitate collected, washed and dried. The yield was practically quantitative; the compound crystallised from glacial acetic acid, or from methylethylketone, in colourless needles, which softened at 195° and melted at 203°.

*Analysis.* 13.9 mg. gave 0.267 mg. N (micro-Kjeldahl).  
0.1482 g. gave 0.1051 g.  $\text{AgI}$ .

	N	I
Found	1.9 %	38.3 %
Calc. for $\text{C}_{26}\text{H}_{21}\text{O}_6\text{NI}_2$	2.1 %	38.0 %

*$\beta$ -[3:5-Diiodo-4-(4'-hydroxyphenoxy)phenyl]- $\alpha$ -aminopropionic acid* (X). 5 g. of the benzoylaminocinnamic ester were boiled under a reflux for one hour with 25 cc. hydriodic acid (Sp. G. 1.7) and 3 g. red phosphorus; the solution was filtered hot through asbestos and evaporated to dryness *in vacuo*: some water was added and the evaporation repeated; the residue was dissolved in warm water with the aid of a little hydrochloric acid, and filtered from some tar. In order to avoid separation of the somewhat insoluble hydrochloride of the amino-acid it was necessary not to cool the acid filtrate more than could be helped; as soon as it was cool enough it was shaken out once with ether, which removed the whole of the coloured impurities; the aqueous solution was then brought to the boil and treated with saturated sodium acetate until no longer acid to Congo red; the amino-acid immediately separated in beautiful silvery platelets, which, after cooling, were filtered off, washed with water, alcohol and ether, and dried. The yield was 1 g. of the

pure substance, or 25 % of the theoretical. The compound gave the ninhydrin reaction, but was too insoluble to permit of a satisfactory determination of amino-nitrogen by the Van Slyke method. It melted at 245–6°.

*Analysis.* 18.1 mg. gave 0.475 mg. N (micro-Kjeldahl).  
0.1270 g. gave 0.1118 g. AgI.

	N	I
Found	2.6 %	47.6 %
Calc. for $C_{15}H_{13}O_4NI_2$	2.7 %	48.3 %

$\beta$ -[3:5-Diiodo-4-(3':5'-diiodo-4'-hydroxyphenoxy)phenyl]- $\alpha$ -aminopropionic acid—*Thyroxine* (XI). 0.525 g. of the diiodoamino-acid was dissolved in 11–12 cc. concentrated ammonium hydroxide (Sp. G. 0.880) and treated, drop by drop, with constant shaking, with *N* iodine in potassium iodide (2 mols.); towards the end of the reaction, the uptake of iodine, which until then had been practically instantaneous, showed a distinct slackening; during the later stages of the reaction a crystalline ammonium salt had begun to separate; after all the iodine had been added, the solution was allowed to stand for some time, and the ammonium salt was then separated on the centrifuge; the salt was dissolved in alcohol with the aid of a little dilute sodium hydroxide, the solution was filtered, boiled and acidified with acetic acid; there separated immediately a colourless crystalline precipitate, which exhibited the very characteristic crystalline form of thyroxine (rosettes and curiously shaped sheaves of colourless needles). This fraction amounted to 0.33 g. A further amount was obtained from the mother-liquor of the ammonium salt as follows: the solution was diluted and boiled until the ammonia was removed; the partly crystalline precipitate which had separated was filtered off and dissolved, so far as possible, in boiling 0.5 % sodium carbonate; the filtered solution, on cooling, deposited a heavy white micro-crystalline precipitate, with the typical appearance of the sodium salt of thyroxine; this was separated on the centrifuge and further treated as described above for the ammonium salt; in this way 0.07 g. of somewhat less well-defined crystalline material was obtained, the total yield, therefore, being 0.4 g., or a little over 50 % of the theoretical. The substance melted at 231° with decomposition; when mixed with a sample of natural thyroxine of m.p. 228°, the mixture melted at 228°; it gave with intensity the colour reaction with nitrous acid and ammonia which is given by thyroxine; further, the solubilities of the natural and synthetic products and of their sodium salts were entirely similar.

*Analysis.* 24.6 mg. gave 0.447 mg. N (micro-Kjeldahl).  
0.99 mg. required 6.08 cc.  $N/200$  sodium thiosulphate [Kendall, 1914].

	N	I
Found	1.82 %	65.1 %
Calc. for $C_{15}H_{11}O_4NI_4$	1.82 %	65.3 %

In so far, then, as the matter is susceptible of decision by chemical methods, the identity of the synthetic product with natural thyroxine may be regarded as established. For the physiological confirmation we are indebted to Prof.

D. Murray Lyon of the University of Edinburgh, a note by whom, on the physiological test, is appended.

We have had the advantage of repeated discussion of the problem with Prof. R. Robinson, F.R.S., and we desire to acknowledge the assistance of Dr C. P. Stewart and of Mr W. McCartney in some of the earliest and latest experiments respectively.

### Note on Physiological Test of Synthetic Thyroxine.

By D. MURRAY LYON.

*Department of Therapeutics, University of Edinburgh.*

*Case 1. Mrs A. S. Age 61. Housewife.*

Known to be myxoedematous in 1921, probably longer. Admitted to hospital in October 1922 showing typical picture of that condition. The patient improved for a time under thyroid extract, but extract of anterior lobe of pituitary did not prevent relapse. Thyroid medication was not continued after discharge from hospital (January 1923).

November 24th, 1926. Third admission to hospital. The patient has taken no care of herself and has never had thyroid extract outside of hospital. She now shows the classical signs of the disease, is dull mentally, is overweight, and has the typical facies and myxoedematous deposits and the harsh dry skin.

The B.M.R. was practically the same as on her previous admission. On November 27th 5 mg. synthetic thyroxine was dissolved in a drop of normal NaOH and made up to 1.5 cc. with distilled water, and was given intravenously. Next day the patient complained of headache and palpitation. By November 29th, two days after the first injection, the B.M.R. had risen from - 32 % to - 17 %. On the 30th the patient herself remarked on her improved condition. She was much brighter and more cheerful, her swellings were diminishing, and wrinkling was obvious on the backs of the hands and under the eyes. Two further doses of 4 and 5 mg. given on November 29th and December 2nd raised the B.M.R. to - 6 % by December 4th. During this period both pulse rate and temperature rose somewhat. A fourth dose given on December 6th seems to have had little effect, as B.M.R., pulse rate and temperature have all declined from about this date. By the 18th the patient had practically returned to her previous condition with B.M.R. - 34 %. The skin had become harsh again and the mental condition much more dull.

The close parallelism between B.M.R. and pulse rate will be noticed in Fig. 1.

*Case 2.* Mrs M. M. Age 35. Housewife.

Normal health until 1916 when she was noticed to be becoming dull mentally and disinclined for effort. Definitely diagnosed myxoedema in 1920. For next few years took thyroid tablets irregularly.

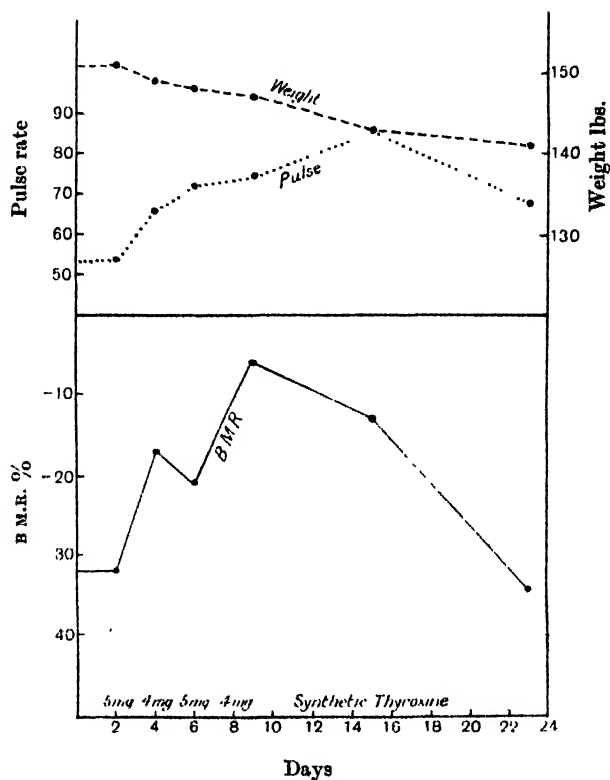


Fig. 1.

January 1926 admitted to Royal Infirmary, Edinburgh, on account of lack of energy and great increase in her swellings. Picture typically that of advanced myxoedema, with a B.M.R. of  $-46\%$ . Remained in hospital from January 5th to March 29th while the dosage of thyroid extract was adjusted. Twelve grains a day proved to be too much and the B.M.R. rose to  $+25\%$ . She was finally discharged on 6 grains a day with a B.M.R. of  $+4\%$ .

Re-admitted November 24th, 1926, not having had any thyroid extract for some months. B.M.R. again  $-45\%$ , the patient being markedly myxoedematous. Three intravenous injections of synthetic thyroxine (5, 4 and 5 mg.) were given on November 27th and 29th, and December 2nd. These produced a marked rise in the B.M.R. to  $+3\%$ , the temperature and the pulse rate rising simultaneously. A fall in weight took place during the same period—partly due to great loss of fluid from the system. A very considerable diuresis followed the first two doses of thyroxine. Each injection also gave rise to an

attack of diarrhoea. As the result of the thyroxine the patient improved remarkably for a time, but after it was discontinued, she slipped back to her former state. In this patient a greater and more lasting effect was produced than in Case 1 (see Fig. 2).

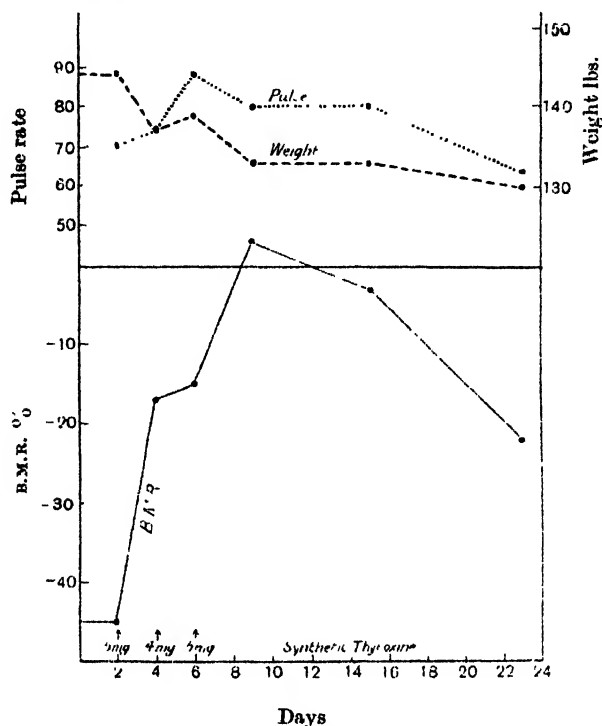


Fig. 2.

The results obtained in the above two cases are shown in Figs. 1 and 2 respectively. The effect of the synthetic thyroxine in raising the basal metabolic rate of these two patients is quantitatively similar to that reported by Boothby and Sandiford [1924] for natural thyroxine.

#### REFERENCES.

- Boothby and Sandiford (1924). *Physiol. Reviews*, **4**, 69.
- Datta and Prosad (1917). *J. Amer. Chem. Soc.* **39**, 141.
- Harington (1926). *Biochem. J.* **20**, 300.
- Kendall (1914). *J. Biol. Chem.* **19**, 251.
- Kendall and Osterberg (1919). *J. Biol. Chem.* **40**, 265.
- Repossi (1916). *Z. Kryst.* **55**, 281.
- Stephen (1925). *J. Chem. Soc.* **127**, 1874.
- Willgerodt and Arnold (1901). *Ber. deutsch. chem. Ges.* **34**, 3344.

## XXIV. ECHINOCHROME.

By ROBERT KEITH CANNAN.

*From the Marine Biological Laboratories, Woods Hole and Plymouth, and the Department of Physiology and Biochemistry, University College, London.*

*(Received December 29th, 1926.)*

ECHINOCHROME is the name which was given by MacMunn [1885, 1889] to a pigment contained in the cells of the perivisceral fluid of *Strongylocentrotus lividus*, *Amphidotus cordatus*, *E. sphaera* and *E. esculentus*. On the grounds of an observed change in the spectrum under the action of strong reducing agents, MacMunn concluded that the pigment was an oxygen carrier. Griffiths [1892] confirmed this opinion but stated that the oxygen was much more firmly held than in oxyhaemoglobin, being removable only by the reducing action of the living cells. Decomposition of the pigment with boiling mineral acids was reported to yield a haemochromogen and a porphyrin. From the elaeocytes of the perivisceral fluid of the deeply coloured sea urchin, *Arbacia punctulata*, McLendon [1912] separated a pigment in a partially purified state, which he found to be spectroscopically identical with the pigment of MacMunn and the name echinochrome was, therefore, extended to it. McLendon found that the red colour of the eggs and of the test of *Arbacia* was due also to the same substance. His preparations contained no significant quantity of iron and he demonstrated that solutions of the pigment which had been submitted to a vacuum did not subsequently absorb any appreciable quantity of oxygen from the air. He consequently questioned the respiratory function of echinochrome.

During a visit to Woods Hole, the writer made the casual observation that the red colour of the aqueous extract of the elaeocytes of *Arbacia* was immediately discharged by sodium hydrosulphite and returned rapidly on shaking with air. In the absence of oxygen, the reduced substance was immediately re-oxidised by mild oxidising agents. The experiment was repeated titrimetrically and the electrode potentials observed. Substantial agreement between the titration curve traced during the reduction of the pigment with standard sodium hydrosulphite and during its re-oxidation with potassium ferricyanide, together with the rapid attainment and constancy of the electrode potentials, indicated that a reversible electrochemical system was under study. A small amount of the crude pigment was prepared and brought back to this country.

## EXPERIMENTAL.

Following, in general, the method of McLendon, the echinochrome was extracted from the test of *Arbacia* with acetone. The extract was evaporated to dryness in a vacuum and the residue extracted with alcohol. Addition of ammonia to the alcoholic solution precipitated the pigment, which was then dissolved in faintly acidified water and the solution extracted with ether. This preparation was far from pure and, as its mass was small, it was, after further fractionation on similar principles, made up to a standard volume in water and this stock solution, preserved on ice, was used for the whole of the work to be reported.

The electrode studies took the usual form of determining the oxidation-reduction titration curve at a series of known  $[H^+]$ ; the detail has been described by Cannan [1926]. Gold plated platinum electrodes were employed, the standard half cell being a saturated calomel electrode. 10 cc. of the echinochrome solution were added to 50 cc. of a buffer [Clark, 1923] of known  $p_H$  and titrated in pure nitrogen with a standard deaerated solution of the oxidising agent similarly diluted with the buffer. The  $p_H$  of each of these mixtures was assumed to be identical with that of the buffer solution diluted to the same extent with water. The latter was determined with the hydrogen electrode. The curves traced by using benzoquinone or ferrieyanide as oxidising agents were identical within the experimental error. Titration of the oxidised pigment with sodium hydrosulphite was vitiated by slow persistent drifts of potential rendering accurate observations impossible. Within the accuracy of observation, however, agreement with the oxidation titration curves was obtained.

The affinity of the reduced pigment for oxygen was so great that much care was necessary to preserve solutions of it from re-oxidation by air. For this reason it was found more certain to repeat the whole titration curve at a series of  $[H^+]$  than to make a series of observations of the potentials of a standard mixture of oxidised and reduced forms added to various buffer mixtures.

Analysis of the experimental curves showed that they were adequately expressed by the electrode equation

$$E_h = E_o' - 0.03006 \log \frac{[S_r]}{[S_o]}$$

where  $E_h$  is the observed potential compared with that of the normal hydrogen electrode, and  $[S_r]$  and  $[S_o]$  are the respective concentrations of the oxidised and reduced forms computed from the titre and the end-point determined graphically.  $E_o'$  is a constant for a given  $[H^+]$ . The value of the numerical factor indicates that the reversible electrode reaction is one involving two equivalents of hydrogen, thus placing echinochrome in the same group as the majority of organic oxidation-reduction systems.

Table I records the abbreviated data of a typical titration curve and indicates that the observations were within the accuracy of the experimental



technique. From the titration curves at other  $[H']$  the values of  $E_o'$  have been deduced and are given in Table II. These serve to define the system over the  $p_H$  range 2 to 7.7 with some certainty.

Table I. *Titration of reduced echinochrome with M/500 potassium ferricyanide.*

$p_H = 3.97$  (10 cc. echinochrome solution added to 50 cc. M/20 acid potassium phthalate).  
Temp. 30°.  $E_o' = -0.0389$ .

Ferricyanide cc.	Oxidation %	$E_h$ (volts)		Deviation
		Found	Calc.	
0.40	2.90	-0.0852	-0.0847	-0.0005
1.00	7.25	0.0722	0.0711	-0.0011
1.60	11.60	0.0659	0.0654	-0.0005
2.20	15.94	0.0604	0.0606	+0.0002
2.80	20.29	0.0567	0.0568	+0.0001
3.40	24.63	0.0534	0.0535	+0.0001
4.60	33.34	0.0481	0.0479	-0.0002
5.80	42.03	0.0429	0.0431	+0.0002
7.00	50.72	0.0387	0.0385	-0.0002
8.20	59.41	0.0339	0.0339	—
9.40	68.11	0.0288	0.0290	+0.0002
10.60	76.81	0.0231	0.0233	+0.0002
11.80	85.51	0.0155	0.0158	+0.0003
12.40	89.84	0.0111	0.0105	-0.0006
13.00	94.19	-0.0034	-0.0026	-0.0008
13.40	97.09	+0.0053	+0.0069	-0.0016
13.80 end point				

The data may be adequately expressed by the equation

$$E_o' = E_o + 0.0601 \log [H'],$$

i.e. the value of  $E_o'$  decreases by 0.0601 v. per unit increase in  $p_H$ . The deviations from the "0.06 slope" at  $p_H$  8.78 and 9.76 may indicate critical acid-base dissociations with ionisation constants in this region. On the other hand, they may be due to the employment of borate buffers, since experience has shown that the presence of borate sometimes leads to anomalous potentials. Neglecting, therefore, these two results, the mean value of  $E_o$  is +0.1995.

Table II. *Relation of  $E_o'$  to  $p_H$ .*

$p_H$	$E_o'$	$0.0601 p_H$	$E_o$
2.21	+0.0668	-0.1328	+0.1996
3.02	+0.0176	0.1815	0.1991
3.97	-0.0389	0.2386	0.1997
4.97	-0.0992	0.2987	0.1995
6.01	-0.1614	0.3612	0.1998
6.75	-0.2056	0.4057	0.2001
7.16	-0.2308	0.4303	0.1995
7.69	-0.2632	0.4622	0.1990
8.78	-0.3220	0.5277	(0.2057)
9.76	-0.3774	0.5866	(0.2092)
Mean			+0.1995

$E_o$  from titration of fresh filtered aqueous extract of elaeocytes (Woods Hole):  
6.74                      -0.201                      -0.405                      +0.204

From a crude experiment at Woods Hole on a fresh aqueous extract of the cells of the perivisceral fluid a value of +0.204 for  $E_o$  was obtained and

another experiment on an extract of the eggs was in close agreement. These results suggest that the pigment had been extracted in an unmodified condition and permit the conclusion that the system which has been defined above actually exists within the cells of *Arbacia*.

Attempts to demonstrate the formation of a compound of reduced echinochrome with molecular oxygen were negative. Moreover, the oxidant produced from reduced echinochrome by oxygen was identical with that formed by mild oxidising agents in the absence of oxygen. This follows from the fact that the experimental titration curves were duplicated by addition to reduced echinochrome of increasing amounts of the pigment formed by atmospheric oxygen.

An attempt was made to confirm these results on the pigment contained in *E. esculentus*. Very little pigment is, however, present and the amount extracted was insufficient for extended study. It was found, that the pigment did represent the oxidant of a reversible oxidation-reduction system but preliminary determinations of the electrode potentials suggested that these were distinctly positive to those of *Arbacia* echinochrome. Moreover, the solubilities of the two pigments differed. These qualitative observations are of interest because it would appear that *E. esculentus* holds its pigment in the partially reduced state, since the perivisceral fluid is almost colourless but rapidly turns red when removed from the animal. In *Arbacia* the echinochrome is in the oxidised state and I know of no observation of the spontaneous decolorisation of the cells *in vivo*. It would be of interest to know if the cells of *Arbacia* can effect a significant reduction of their pigment anaerobically—bacteria and yeast will slowly reduce it in the absence of oxygen—but the author has no longer access to the biological material.

The rate of oxidation of reduced echinochrome by atmospheric oxygen was too great to be measured by any simple means.

#### DISCUSSION.

If any respiratory rôle is to be attributed to echinochrome it is clear that it must be that of an "activator" rather than of a "carrier" of oxygen. It forms no dissociable compound with molecular oxygen. It is of interest to consider this possibility briefly in the light of the quantitative data presented since it represents a concrete case of a type of respiratory catalyst frequently postulated. At the same time it must be acknowledged that the function suggested for echinochrome is at present without physiological proof.

It is probable that within living cells there exists a dynamic equilibrium—an oxidation-reduction poise—between the inherent reducing activity of the cells and the supply of active oxygen [Cannan, Cohen and Clark, 1926]. A characteristic reduction potential may be attributed to the cell, and the state of oxidation of the echinochrome present will be conditioned by this. If the assumption be made that the pigment is the effective oxygen activator then the rate of oxygen consumption of the cell will be determined by the con-

centration of reduced pigment in equilibrium—since its oxidation by molecular oxygen is very rapid. It is improbable that the cells can, aerobically, achieve a reduction intensity (corresponding to  $r_H$  7) sufficient to reduce a measurable fraction of the total pigment present but it is clear from the electrode equation that at reduction intensities much less than this there remains a concentration of reduced pigment which, whilst analytically insignificant, may be a sufficient molecular concentration to insure a measurable oxygen consumption.

One further implication is of interest. It is well known that fertilisation of the egg of the sea urchin is followed by an enormous increase in the oxygen consumption. J. and D. Needham [1926] failed to observe a coincident increase in the reduction potential of the egg by their method of the micro-injection of indicators, although such an increase was to be expected. Now a fall in  $r_H$  of only one unit (an increase in reduction potential of 60 millivolts) would lead to a tenfold increase in the concentration of reduced echinochrome present. The method of injection of indicators was, in the present case, incapable of observing any change in  $r_H$  less than two or three units so that it was possible for a change to have occurred corresponding to an increase of several hundredfold in the concentration of reduced pigment, and, therefore, of oxygen consumption. It will be seen that in such a hypothetical system there is wide metabolic latitude although the system is well poised. Its investigation, however, requires exact information of the rate of oxidation of reduced echinochrome and of the reduction potential of the cell under the experimental conditions.

It has frequently been recorded that in the eggs of *Arbacia* fertilisation is followed by a migration of the chromatophores to the periphery of the cell. This might be considered to be an accessory, or alternative, means for increasing the concentration of reduced pigment in effective contact with oxygen. On the other hand, though this is improbable, the supposed migration may mask an actual reduction of the echinochrome in the interior of the cell owing to an enhanced reduction intensity following fertilisation.

One final qualification must be made. We have no present means of deciding to what extent the homogeneous equilibria represented by electrode potentials are modified in a heterogeneous system such as the living cell. This is particularly cogent in the present case where the pigment exists in granules microscopically distinguishable as a separate phase. The physiological application of electrometric data must be made with caution.

#### SUMMARY.

1. Echinochrome has been separated from the eggs, perivisceral fluid and test of *Arbacia punctulata*. It does not form a dissociable compound with oxygen but is the oxidant of a reversible oxidation-reduction system.
2. The electrode potentials of the system have been measured over the  $p_H$  range 2.2-9.76. The normal electrode potential is + 0.1995.
3. The alleged respiratory function of echinochrome is discussed.

For the hospitality of the Marine Biological Laboratories of Woods Hole and of Plymouth the author is indebted to Dr F. Lillie and to Dr E. G. Allen respectively.

Acknowledgement is made of a grant from the Government Grant Committee of the Royal Society from which a part of the expenses of this work have been defrayed.

## REFERENCES.

- Cannan (1926). *Biochem. J.* **20**, 927.  
Cannan, Cohen and Clark (1926). *U.S. Public Health Rep. Suppl.* 55.  
Clark (1923). *The Concentration of Hydrogen Ions* (2nd edition).  
Griffith (1892). *Physiology of the Invertebrata* (New York).  
MacMunn (1885). *Quart. J. Micro. Sci.* **28**, 469.  
—— (1889). *Quart. J. Micro. Sci.* **30**, 51.  
McLendon (1912). *J. Biol. Chem.* **11**, 435.  
Needham, J. and Needham, D. (1926). *Proc. Roy. Soc. Lond. B*, **99**, 173.

## XXV. THE INORGANIC PHOSPHATE AND A LABILE FORM OF ORGANIC PHOSPHATE IN THE GASTROCNEMIUS OF THE FROG.

BY PHILIP EGGLETON AND GRACE PALMER EGGLETON.

*From the Department of Physiology and Biochemistry,  
University College, London.*

*(Received December 31st, 1926.)*

IN a study of the significance of phosphorus in muscle contraction (which will be reported separately) some observations were made which throw a doubt on the chemical results of some earlier workers. Evidence is given in this paper to show that the supposed inorganic phosphate of muscle is in certain conditions mainly organic phosphate of a very labile nature, which is so unstable in acid solution that it is hydrolysed during the estimation of the inorganic phosphate by the methods of Neumann, Embden [1921] or Briggs [1922]. Estimations performed in neutral or slightly alkaline solution, such as the Bell-Doisy method [1920], or precipitation by magnesia mixture, give results approximating to the truth, provided the muscle extract has not previously been exposed to the action of acid.

Briggs' method itself can be used to demonstrate the existence of this unstable phosphoric ester. Details of the method are given elsewhere: it suffices for the moment to say that after the addition of the appropriate reagents to a solution containing phosphate a blue colour develops, which rises to a maximum in about 30 minutes, after which time colour comparisons are usually made. It is easy to show that the rate of development of this blue colour follows a simple exponential law,

$$\text{intensity at time } t = c = P(1 - e^{-kt}),$$

where  $P$  is the final colour which measures the phosphate. The constant  $k$  has the value 0.12 (time measured in minutes). It follows mathematically (and can be proved experimentally) that in comparing two quantities of inorganic phosphate the ratio of the colour intensities will be the same at whatever time the comparison is made. If the colour ratio be plotted against time the result is a straight line parallel to the time axis (lines  $AB$  and  $CD$  in Fig. 1). A deproteinised extract from a dead muscle (killed by warming to  $35^\circ$  for 20 minutes) compared in this manner against an inorganic phosphate solution gives also a straight line, and is indistinguishable in this respect from an inorganic phosphate solution. When however a similar extract from a resting muscle is compared against an inorganic standard a different result

is obtained. The colour development begins by appearing equivalent to a very weak standard, but rapidly overtakes a much stronger standard. The result is a curve such as the curves *R* and *R'* in Fig. 1.

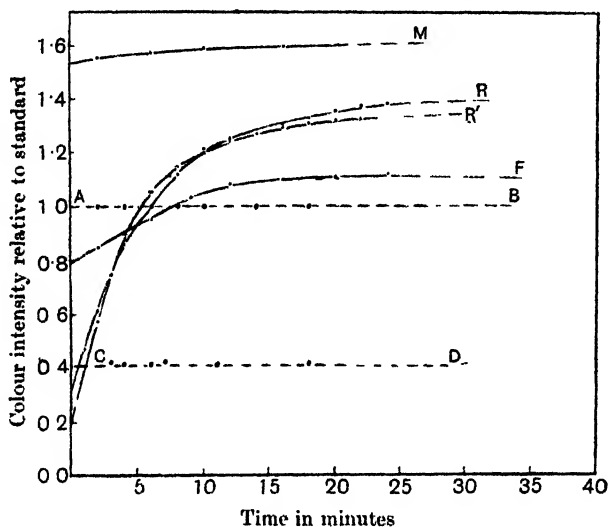


Fig. 1. The inorganic phosphate solution which gave the line *AB* contained 0.1 mg. of phosphorus. Another solution containing 0.04 mg. gave the parallel line *CD*. The remaining curves were given by muscle filtrates corresponding in each case to 160 mg. of muscle. The lines represent the colour intensity at different times relative to the standard solution which gave the line *AB*. (*R* and *R'*, resting; *F*, fatigued; *M*, in rigor.)

So complex are the conditions of colour development that little importance would attach to this fact were it not that a fatigued muscle shows this effect to a much less extent (curve *F*), whilst as has been said already, a muscle in rigor mortis shows no such effect at all (line *M*). Of the possible explanations which present themselves, the only one which survives the test of experiment is that there is an organic phosphate breaking down during the course of the estimation, causing the colour development curve to be pushed higher and higher. That this substance is completely destroyed in 20 minutes or so is shown by the curves in Fig. 2. In this type of experiment two portions of a resting muscle filtrate are treated simultaneously with Briggs' acid molybdate solution, but in one case the reducing solution is not added until 30 minutes later. In the latter case the colour production curve runs strictly parallel with an inorganic standard. The unstable organic phosphate has already broken down.

As to the nature of this unstable compound we have no evidence, save that it is concerned in muscular activity, and its disappearance coincides with fatigue. The name "phosphagen" suggests itself. "Phosphagen" might possibly be a phosphoric ester of glycogen, for traces of glycogen are present in trichloroacetic acid extracts of muscle. It is also possible that it may be the substance postulated by Meyerhof as a precursor for both lactic acid and "lactacidogen."

When Briggs' method is used to estimate the true inorganic phosphate, all that is necessary is to make colour comparison with the standard at frequent intervals, and obtain a curve such as those in Fig. 1. By extrapolating this curve backwards to zero time a value is obtained which represents the colour ratio which would have been given at the end of 30 minutes had the "phosphagen" been stable. The difference between this true value and that obtained after 30 minutes, measures the "phosphagen" in terms of the inorganic phosphate formed by its decomposition.

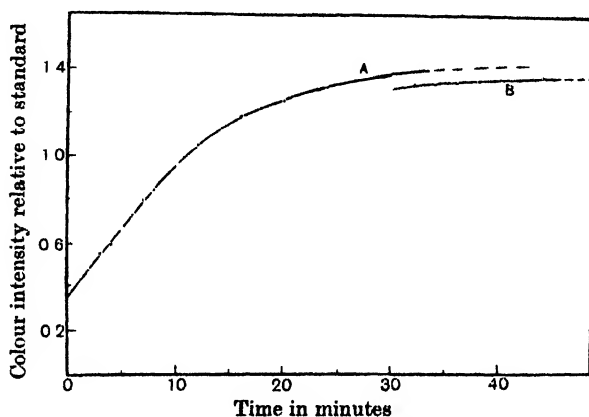


Fig. 2. The curve *A* represents the colour intensity at different times (relative to an inorganic standard started off at the same moment) given by an extract from a resting muscle, when the "inorganic" phosphate is estimated by Briggs' method. If the reducing agent is added 30 minutes after the acid molybdate reagent the colour production curve (*B*) is such as would be given by true inorganic phosphate. The "phosphagen" is already broken down. (The starting-point of the curve *A* is a measure of the true inorganic phosphate, whilst the height to which the curve rises is a measure of the "phosphagen.")

Table I. *Showing the rapid breakdown of "phosphagen" in acid solution.*

A fresh trichloroacetic acid filtrate from a resting muscle was estimated for inorganic phosphate and "phosphagen." Part of the filtrate was neutralised with  $\text{NaHCO}_3$  and the remainder not treated. Both portions were incubated for an hour at  $27^\circ$  and the estimations were repeated on each. Results given in mg. of phosphorus per 100 g. of muscle.

	Inorganic P	"Phosphagen"	Increase in inorganic P	Decrease in "phosphagen"
Initial	27	57	—	—
After 1 hour at $27^\circ$	51.5	33	24.5	24
Incubated in presence of $\text{NaHCO}_3$	30.5	50	3.5	7

We have used 4 % trichloroacetic acid as a deproteinising agent, and have found that even in this solution the unknown substance disappears in a day or two. So rapid is this breakdown in the first hour or so, that special precautions must be taken to perform estimations as soon as possible after the removal of protein. Alternatively the extract can be neutralised with sodium bicarbonate, which renders the "phosphagen" less liable to breakdown on standing (Table I).

The stability of "phosphagen" in neutral solution led us to try the estimation of inorganic phosphate by the Bell-Doisy method, which is performed

in a mildly alkaline solution. The results (see Table II) agreed closely with the "corrected" values obtained by Briggs' method. In passing it should be mentioned that Bell and Doisy probably found traces of "phosphagen" in blood, for they state that "the inorganic phosphate must be determined in the trichloroacetic acid filtrate as soon as possible after filtering since the acid hydrolyses the organic phosphorus on standing, giving too high results. After standing 24 hours the values for inorganic phosphate and the total acid-soluble phosphorus are nearly identical." In the case of blood however the discrepancy appears to be a small one—not more than about 10 % of the value quoted for inorganic phosphate. In resting muscle extracts we have a discrepancy of the order of 300 %.

Estimation of the inorganic phosphate with magnesia mixture confirmed our belief that we were dealing with an organic phosphate unstable in acid. Although this method is notorious for giving high results (on account of the simultaneous precipitation of calcium and organic matter), our figures indicate that a resting muscle contains not more than 40 mg. % of inorganic phosphorus, less than half the figure given by the method of Embden (or any method involving the use of mineral acids).

Table II. *The "inorganic phosphate" of the frog's gastrocnemius, showing the falsely high values given by the Briggs and Embden methods, particularly for resting muscles.*

Exps. A, D, E and K were performed on batches of 6 to 8 frogs. In Exps. E, F and G the left gastrocnemius was used as a resting control on the right, which was stimulated through the nerve for 2 to 5 minutes with a supermaximal stimulation. The Briggs and Embden methods give high resting values which fall in fatigue. The other methods give low values which rise considerably in fatigue. All methods give the same high value for rigor.

"Inorganic P" in mg. per 100 g. of muscle

		Embden	Briggs	Briggs corrected	Bell-Doisy	Magnesia mixture
Resting	A	92	87	—	—	—
	B	—	83	—	22	—
	C	—	91	18	—	—
	D	—	88	—	—	40
	E	95	86	—	—	—
	F	—	87	33	—	—
	G	—	84	28	27	—
Fatigued	K	—	90	22	28	12
	E	75	72	—	—	—
	F	—	73	56	—	—
Rigor	G	—	68	45	52	—
	H	—	105	102	—	—
	I	—	96	102	—	—
	K	—	104	90	108	103

#### EXPERIMENTAL.

The muscles were killed by immersion in liquid air and ground up with 4 % trichloroacetic acid in a mortar. The fluid was washed into an accurately calibrated cylinder and the volume made up with 4 % trichloroacetic acid to 4 or 5 cc. per 100 mg. of muscle. It was found essential to keep the temperature down to 0° until the actual estimation was performed; moreover the



fluid was filtered within 10 minutes of the maceration. Contrary to the results of certain other workers we have found it quite unnecessary to allow the extraction to continue overnight: extraction in 3 minutes was almost as complete as in 20 hours.

A quantity of filtrate calculated to contain about 0.1 mg. of phosphorus (inorganic plus "phosphagen") was diluted in a 15 cc. graduated flask to exactly 12 cc. An inorganic standard of equal or less strength contained in a similar flask was treated with the same amount of 4 % trichloroacetic acid and similarly diluted. The two reagents were added<sup>1</sup>, and the solutions after being mixed were poured directly into the colorimeter cups. Readings could in this way be made within the first minute of the period of colour production. It is essential in following the colour production curves that the unknown and standard should be started off simultaneously and should be at the same temperature.

Where the Embden technique was used Embden's directions were followed in every detail, save that trichloroacetic acid was used to deproteinise the muscles. This is a simpler method and gives the same results as the Schenck method recommended by Embden.

The Bell-Doisy method was modified slightly to meet our special requirements. Instead of allowing 5 minutes for their acid-molybdate-quinol solutions to act before the addition of the sulphite-carbonate reagent, we reduced the time to about 1 minute for both muscle extract and inorganic standard.

The magnesia mixture used contained magnesium citrate instead of the chloride. This is supposed to give truer values.

#### DISCUSSION.

We have found that the true inorganic phosphate content of a resting frog's gastrocnemius is of the order of 20 to 25 mg. of phosphorus per 100 g. of muscle. Estimations performed by the Briggs or Embden method give results of the order of 90 to 100 mg. per 100 g. in our hands (Embden's own figures are rather higher). This discrepancy of 70 mg. per 100 g. is attributable to a phosphoric ester which is very unstable in acid solution, and which is estimated as inorganic phosphate by the above methods. We have called this new compound "phosphagen."

In rapidly induced fatigue the true inorganic phosphate content is doubled at the expense of the "phosphagen," but considerably more "phosphagen" disappears than is necessary to account for the rise in inorganic phosphate. It is obvious that a method which estimates both inorganic phosphorus and "phosphagen" together should show a fall as a result of fatigue. We have found such a fall by both Embden's and Briggs' methods, though Embden

<sup>1</sup> Solution A: 5 % ammonium molybdate in 5.4 N sulphuric acid. Solution B: 0.5 % quinol in 20 % sodium sulphite. Solution B reduces the phosphomolybdate formed by the interaction of solution A with the inorganic phosphate.

himself claims to have found a rise [Embden *et al.*, 1922, 1925, 1926]. The figures quoted by Embden are so high, for both resting and fatigued muscles, that they cannot refer to the true inorganic phosphate.

When a muscle goes into rigor the "phosphagen" disappears entirely, and the inorganic phosphate rises to about four times the resting value. Here the "phosphagen" is more than accounted for by the inorganic phosphate which appears.

We desire to express our thanks to Prof. A. V. Hill and to Dr R. Robison for their suggestive criticism during the progress of the work, and to the Medical Research Council for a grant to meet the expenses.

#### REFERENCES.

- Bell and Dossy (1920). *J. Biol. Chem.* **44**, 55.  
Briggs (1922). *J. Biol. Chem.* **53**, 13.  
Embden (1921). *Z. physiol. Chem.* **113**, 139.  
Embden and Hentschel (1925). *Biochem. Z.* **156**, 343.  
——— (1926). *Z. physiol. Chem.* **151**, 167.  
Embden and Lawaczek (1922). *Biochem. Z.* **127**, 184.

# XXVI. NOTE ON THE PREPARATION OF YEAST-JUICE BY BUCHNER'S METHOD.

BY ARTHUR HARDEN AND FRANCIS ROBERT HENLEY.

*From the Biochemical Department, Lister Institute, London.*

*(Received December 31st, 1926.)*

SOME years ago (1920), before the yeast obtainable from breweries had regained its pre-war qualities, considerable difficulty was experienced in obtaining yeast-juice of a satisfactory degree of activity. It was, however, observed that, when the ground mass was kept for a few hours before being pressed out, the resulting juice was more active than that obtained by pressing at once. Accordingly a few experiments were made to test the reality of this effect and to ascertain under what conditions it was produced.

It was found that as a rule the juice obtained by pressing after an interval of 1-2 hours reacted much more vigorously with added phosphate than that pressed out at once.

This effect has been recently confirmed, although the yeast-juice obtained by direct pressing on this occasion was much more active than that prepared six years ago. Accordingly the preservation of the ground mass at air temperature for 2 hours before it is pressed out has been adopted as part of the routine of the preparation. Preservation of the expressed yeast-juice does not produce the same effect, so that it is probably due to some action involving constituents of the cell which are retained in the press cake. This question is being further investigated. All the experiments have been made with top-yeast from an English brewery.

*Exp. 1.* The ground mass was kept at air temperature and pressings made immediately after grinding and at intervals of 22 mins., 42 mins., 1 hr. 42 mins., 2 hrs. 42 mins., and 3 hrs. 42 mins. after grinding. Some of the juice from the first pressing was also kept at air temperature for 2 hours and then examined.

In each case two quantities of 25 cc. yeast-juice + 1 g. glucose + 0.2 cc. toluene were incubated at 25° for 35 mins. and the rate of fermentation observed. 2.5 cc. 0.6  $M$   $K_2HPO_4$  + 2.5 cc.  $H_2O$  were then added to one of these quantities and 5 cc. 0.6  $M$   $K_2HPO_4$  to the second and the rate of fermentation again observed.

The results were as follows:

Interval between grinding and pressing	After addition of					
	Initial rate cc. per 5 mins.	2.5 cc. 0.6 $M$ $K_2HPO_4$			5 cc. 0.6 $M$ $K_2HPO_4$	
		Max. rate cc. per 5 mins.	Total in 45 mins.		Max. rate cc. per 5 mins.	Total in 45 mins.
0	1.6	3	23.4		2.4	11.5
22 mins.	3.3	4.6	29.8		3	24.5
42 mins.	3.1	4.6	34		8.7	35.6
1 hr. 42 mins.	3.6	6.1	44.8		5.6	36.2
2 hrs. 42 mins.	2.8	6.1	39.8		4.6	37
3 hrs. 42 mins.	3	5	40.9		3.6	29
Juice preserved for 2 hrs.	1.6	3.2	20.6		2.4	13.4

*Exp. 2.* The experiment was similar to *Exp. 1* but only two pressings were made and three different quantities of phosphate (in this case a mixture of 8 vols. of 0.6 *M*  $\text{KH}_2\text{PO}_4$  with 2 of 0.6 *M*  $\text{K}_2\text{HPO}_4$ ) were used.

Interval between pressing and grinding	Initial rate cc. per 5 mins.	No addition Total in 45 mins.	After addition of phosphate					
			5 cc.		10 cc.		15 cc.	
			Max. rate	Total in 45 mins.	Max. rate	Total in 45 mins.	Max. rate	Total in 45 mins.
0	1.65	8.2	9.5	78.3	5.4	46.4	4.8	28.6
2 hrs.	1.5	10.8	19.3	85.8	16.1	111.8	8.3	61

## SUMMARY.

In preparing yeast-juice by the grinding method from English top-yeast it is usually advantageous to preserve the ground mass for two hours at air temperature before pressing out the juice.

## XXVII. THE AEROBIC AND ANAEROBIC METABOLISM OF THE COMMON COCKROACH (*PERIPLANETA ORIENTALIS*). II

By WILLIAM KERSHAW SLATER (*Beit Memorial Research Fellow*).

*From the Department of Physiology and Biochemistry,  
University College, London.*

*(Received December 31st, 1926.)*

It has recently been shown [Davis and Slater, 1926] that when the common cockroach (*Periplaneta orientalis*) is placed in an atmosphere of nitrogen or some other inert gas, it goes into debt for oxygen, in a manner similar to that which has been observed in the case of striated muscle. This phenomenon was thought to be worthy of further investigation, in the hope that the superficial similarity might prove to be the expression of a common fundamental metabolism.

The metabolic process involved in the case of vertebrate muscle has been shown by Hill, Meyerhof and their co-workers to consist of two separate reactions, viz. (1) an initial breakdown of glycogen to lactic acid, and (2) the transformation of a portion of the lactic acid back again into glycogen, at the expense of the combustion of the remainder. The first reaction is anaerobic, and is the source of energy in the organism, the second is aerobic, and serves simultaneously as a means of removing the lactic acid, and of conserving the energy available in the glycogen. The efficiency of the total process depends largely upon the ratio of the lactic acid returned to glycogen to the lactic acid burnt. During oxygen lack the tissue obtains its energy in the normal manner, the lactic acid being allowed to accumulate. When oxygen again becomes available, this lactic acid is removed by synthesis and oxidation, the organism using an excess of oxygen, equal to that which it would have used during the period of anaerobiosis. In the case of vertebrate muscle, the ratio of lactic acid burnt to that resynthesised to glycogen is given by Furasawa and Hartree [1926] as 1:4.4, from which it can be calculated that a debt of 1 cc. of oxygen corresponds approximately to 6 mg. of lactic acid.

Working upon the assumption that a similar process is involved in the metabolism of the cockroach, it is possible, knowing the oxygen debt, to calculate (1) the lactic acid which should be found in the tissue, and (2) the volume of carbon dioxide which would be evolved if the lactic acid were completely neutralised by carbonate. This latter figure will be identical with the carbon dioxide which is retained when the lactic acid is subsequently removed.

An investigation is at present being carried out with a view to estimating the lactic acid occurring in conjunction with a given oxygen debt after a period of anaerobiosis. The present communication deals with a more detailed study of the respiratory exchanges following anaerobiosis in order to show, firstly, whether carbon dioxide is retained, and secondly, if this proves to be the case, whether the volume of the retained gas bears any relationship to the calculated amount of lactic acid present.

It may appear at first sight that it would be simpler and more direct to measure the carbon dioxide evolved during anaerobiosis, but practically it is easier to compare over a given recovery period the excess oxygen used and the carbon dioxide retained.

### METHOD.

The measurements were made by means of the micro-respiration apparatus previously described [Slater, 1926]. This enabled the oxygen uptake and the carbon dioxide output to be determined simultaneously with an accuracy greater than 1 mm.<sup>3</sup>.

Since the apparatus was originally described, some alteration has been made in the method adopted for ascertaining changes in the volume of the gases. The respiration vessel, in which the carbon dioxide is measured electrically by means of the Shakespear katharometer, is now connected to a small open manometer, instead of the original Haldane blood gas apparatus. At the bottom of the U of the manometer is a small rubber bag, which can be compressed by means of a plate and thumb screw. As the volume of the gases in the respiration chamber alters, the bag is compressed or relaxed, so as to bring the liquid on each side of the U back to the same level. By calibrating the arm of the U on the closed side, the changes in volume may be read at atmospheric pressure. To avoid errors which may be introduced during the experiment, by changes in barometric height or in the temperature of the thermostat, a second bulb as similar to the respiration vessel as possible and connected to a similar manometer, is placed in the thermostat alongside the actual apparatus. The changes in the volume of the air in this vessel are applied as a correction to the actual readings obtained for changes in the volume of the respired gas mixture. The new manometric arrangement has the advantage that it is small, and can be sealed directly on to the tube from the respiration vessel, thus avoiding all rubber joints, and permitting, if necessary, that the whole apparatus be shaken. This method of measuring volume changes is based upon that described by Warburg [1923].

The cockroaches were caught in the building, and were used immediately (except in those cases where they were kept for a few days on a glucose diet in order to raise their respiratory quotient) to ensure that they were in no way damaged by being kept in captivity. The respiratory quotient of the freshly caught insects was rarely above 0.85, probably due to the fact that

in the building where they were caught, there was abundant food of fat and protein, but hardly any carbohydrate.

The insect selected for the experiment was weighed, and dropped into the lower part of the respiration vessel. Care was taken in attaching this to the upper portion that the antennae were not trapped in the ground glass joint. The apparatus was then placed in a thermostat kept at 25°, and left until the cockroach had settled into a quiet state. When the necessary conditions had been attained an oxygen-carbon dioxide mixture was led through the apparatus until the reading of the katharometer showed that all the air had been expelled. The taps were then closed and readings taken of the carbon dioxide percentage and the total volume. When the readings became steady they were continued for an hour in order to get a value for the resting metabolism under the conditions of the experiment. A stream of nitrogen was next passed through the apparatus until the cockroach ceased to struggle. The vessel was then closed for from 30 mins. to 1 hour. As will be seen later, for purposes of comparison it is not necessary that the anaerobiosis should be absolute. Such a condition would be difficult to obtain with the type of apparatus which was in use. At the end of the anaerobic period the nitrogen was rapidly replaced by the oxygen-carbon dioxide mixture, and readings commenced as soon as possible and continued until the respiratory quotient again reached its normal value.

#### RESULTS.

In Table I are given the chief details of 7 experiments.

Table I.

Experiment	1	2	3	4	5	6	7
Weight of cockroach (g.)	0.13	0.07	0.19	0.22	0.18	0.12	0.08
Condition of cockroach	Glucose-fed	Freshly caught	Freshly caught	Freshly caught	Freshly caught	(Glucose-fed)	Starving for 1 month
% carbon dioxide at the beginning of the experiment	1.70	1.76	1.44	1.47	0.83	0.60	0.35
Normal respiration:							
Oxygen mm. <sup>3</sup> per g. per hour	314	375	292	258	398	366	225
Carbon dioxide mm. <sup>3</sup> per g. per hour	315	365	242	241	277	342	200
Post anaerobic respiration (period of excess oxygen intake):							
Oxygen mm. <sup>3</sup> per g. per hour	370	433	350	359	458	416	288
Carbon dioxide mm. <sup>3</sup> per g. per hour	262	384	249	260	305	346	209
Excess oxygen used mm. <sup>3</sup> per g. per hour	56	58	58	101	60	50	63
Carbon dioxide retained mm. <sup>3</sup> per g. per hour	108	39	51	82	32	46	54

In the table the rise in oxygen intake after anaerobiosis is clearly marked in each experiment, but this value is not in proportion to the time during which the cockroach was in nitrogen. This is due to the difficulty experienced

in obtaining the complete removal of oxygen by the method used. The nitrogen always contained traces of oxygen, and the shape of the respiration vessel rendered the complete removal of the gases previously present a matter of considerable difficulty. As however the results are used only for the comparison of the oxygen debt replacement and the carbon dioxide retention over the same period of recovery, the efficiency of anaerobiosis is immaterial, provided always that it does not vary outside those limits which may be expected to produce profound tissue derangement.

It was shown [Davis and Slater, 1926] that the normal oxygen uptake at 25° was about 500 mm.<sup>3</sup> per g. per hour. In the present experiments the values obtained are all below this average figure. No positive explanation of this difference can be advanced, but it is tentatively suggested that it is due to the fact that the present experiments were carried out at a definite carbon dioxide pressure. Enough data are not available in support of this view to render it more than a working hypothesis, although the results of the experiments in Table I agree with it as far as they have been carried. With the exception of Exp. 7, where the cockroach was distinctly abnormal, owing to being without food for over a month, the oxygen intake appears to be reduced with higher percentages of carbon dioxide. (It is to be noted that in Exp. 7, the weight of the insect is only 0.08 g., *i.e.* it is not fully matured and hence according to previous determinations its oxygen uptake should be approximately 600 to 700 mm.<sup>3</sup> per g. per hour.)

To obtain the carbon dioxide retention during the post anaerobic phase, it is necessary to calculate the carbon dioxide which should have been evolved during this period. This value is easily obtained if we assume that the normal metabolism is going on with the recovery process superadded; for under these conditions the carbon dioxide evolved will be equal to the normal plus that from the oxidation of the lactic acid. The combustion of the lactic acid has a unit respiratory quotient, and hence the carbon dioxide evolved is equal to the excess oxygen taken in. Thus we have

$$(\text{CO}_2 \text{ output after anaerobiosis} = \text{Normal CO}_2 \text{ output} + \text{O}_2 \text{ debt.})$$

The carbon dioxide retained, as given in the last row of figures in Table I is obtained by subtracting the output found from the output calculated in this way. The retention of carbon dioxide is clearly shown in each experiment. In Table II are shown the respiratory quotients for the same experiments, again illustrating the carbon dioxide retention.

Table II.

Experiment	1	2	3	4	5	6	7
Normal R.Q.	1.00	0.98	0.83	0.93	0.70	0.93	0.89
Post anaerobic R.Q.:							
Calculated	1.00	0.98	0.86	0.95	0.73	0.94	0.91
Found 1st half hour	0.60	0.87	0.72	0.65	—	—	—
2nd "	0.83	0.91	0.68	0.63	0.62	0.82	0.53
3rd "	1.03	0.98	0.73	0.74	0.71	0.83	0.95
4th "	0.96	0.97	0.83	0.83	0.81	—	0.78



It will be seen that in the majority of cases, the respiratory quotient has returned to normal at the end of 2 hours. The figures given for the calculated respiratory quotient for the post anaerobic period allow for the excess oxygen used liberating its own volume of carbon dioxide.

There remains now only the consideration of the relation between the oxygen debt and the carbon dioxide retained. If the figure given above—1 cc. of oxygen equivalent to 6 mg. of lactic acid—is used to calculate the lactic acid present in the tissue, the results shown in row 3 of Table III are obtained.

Table III.

Experiment	1	2	3	4	5	6	7
Excess oxygen mm. <sup>3</sup>	56	58	58	101	60	50	63
Lactic acid corresponding to excess oxygen, mg.	0.336	0.348	0.348	0.606	0.360	0.300	0.378
Carbon dioxide retention calc. from lactic acid, mm. <sup>3</sup>	84	87	87	151	90	75	94.5
Carbon dioxide retention found, mm. <sup>3</sup>	108	39	51	82	32	46	54
Ratio CO <sub>2</sub> found/CO <sub>2</sub> calc.	1.2	0.46	0.59	0.54	0.36	0.60	0.57

From the lactic acid, the volume of carbon dioxide which should be retained during the recovery process can be calculated, and is given in row 4. The accuracy of these figures depends on two unknown factors, viz. (1) the extent to which carbonates are involved in the neutralisation of the lactic acid, and (2) the efficiency of the oxidative recovery process. For purposes of the calculation it has been assumed that the whole of the neutralisation is by carbonates, and that the efficiency of the recovery process is the same as that of striated muscle. It is improbable that the first assumption is correct, and there is no precedent for the second. Fortunately these unknown factors will affect all the results proportionately, and if we determine the ratio between the carbon dioxide found and that calculated, it should, on the assumption that the carbon dioxide retention is due to the removal of the lactic acid, be approximately constant. This ratio is given in row 5, and, with the exception of Exp. 1, the values lie between 0.36 and 0.60, with a mean of 0.52, and a percentage mean deviation of 14.6. For the type of experiment under consideration, the agreement is sufficiently good to lend support to the view that the oxygen debt and the carbon dioxide retention are related through the removal of accumulated lactic acid. In the case of Exp. 1 it can only be suggested that some unknown error has been introduced, but as it was the only experiment which fell outside the series, it was considered reasonable to neglect it for the time being.

## SUMMARY.

(1) After a period of anaerobiosis the cockroach not only uses an excess of oxygen, but also retains a considerable volume of carbon dioxide.

(2) The oxygen debt and the carbon dioxide retention are shown to be related through the removal of lactic acid.

The expenses of this research were largely defrayed by a grant from the Royal Society.

## REFERENCES.

- Davis and Slater (1926). *Biochem. J.* **20**, 1167.  
Furasawa and Hartree (1926). *J. Physiol.* **62**, 203.  
Slater (1926). *J. Sci. Instruments*, **3**, 177.  
Warburg (1923). *Biochem. Z.* **142**, 317.

## XXVIII. SOME PHOSPHORUS COMPOUNDS OF MILK. III.

### DEPHOSPHORISED CASEINOGEN. THE ACTION OF ALKALI UPON CASEINOGEN.

By CLAUDE RIMINGTON

(*Benn W. Levy Student: Research Scholar of Emmanuel College, Cambridge.*)

*From the Biochemical Laboratory, Cambridge.*

(*Received December 31st, 1926.*)

THE name "dephosphorised caseinogen" was given by Rimington and Kay [1926] to the protein produced from caseinogen by the action of 0.25 N sodium hydroxide for 24 hours at 37°. This substance contains only minimal amounts of phosphorus but in other respects does not differ greatly from caseinogen. Preliminary experiments indicated, however, that dephosphorised caseinogen possesses a low nitrogen content, values lying between 11.1 % and 13.1 % being found for samples prepared from different brands of commercial "casein," the total nitrogen of caseinogen itself being 15.65 %.

During the reaction with sodium hydroxide a considerable evolution of ammonia occurs and it was with the object of ascertaining the source of this ammonia that the following investigations were undertaken.

Dephosphorised caseinogen, obtained from a specimen of caseinogen prepared from separated milk, was analysed by the nitrogen distribution method of Van Slyke, employing the modifications recently suggested by Plimmer and Rosedale [1925]. The analyses were performed in duplicate. In Table I are

Table I. *Distribution of nitrogen in dephosphorised caseinogen.*

Total nitrogen = 11.06 %, total phosphorus = 0.009 %.

	Error of the method (Van Slyke) % of total nitrogen	Caseinogen				Dephos- phorised caseinogen	Dephos- phorised caseinogen	Caseinogen as
		Crowther and Raisstrick % of total nitrogen	Plimmer and Rosedale % of total nitrogen	Van Slyke % of total nitrogen	Caseinogen Mean of last three columns	Mean of two analyses % of total nitrogen	N - amide N	percentage of total amide N
Amide-N	0.37	10.25	10.63	10.35	10.41	4.35	—	—
Humin-N	0.39	1.26	1.08	2.35	1.56	1.80	1.88	1.74
Total N of bases	—	26.62	22.68	22.85	24.03	27.25	28.49	26.84
Arginine-N	1.27	9.31	7.74	7.46	8.17	6.82	7.13	9.12
Histidine-N	0.93	6.55	5.54	5.23	5.77	7.03	7.35	6.44
Lysine- + cystine-N	1.23	10.76	9.41	10.16	10.11	13.39	14.01	11.28
Total N of filtrate	1.50	62.31	62.58	63.75	62.88	64.13	67.05	70.16
Amino-N of filtrate	1.10	55.44	51.93	55.43	54.27	60.21	62.95	60.56
Non-amino-N of filtrate	1.20	6.87	10.66	8.32	8.62	3.92	4.10	9.60

recorded the mean results expressed as percentages of total nitrogen, together with some previous analyses of caseinogen to be found in the literature. The figures given by Plimmer and Rosedale have been recalculated for expression as percentages of total nitrogen. In order further to facilitate the comparison of dephosphorised caseinogen with caseinogen, the nitrogen distribution in these two proteins has also been calculated in such a way that the nitrogen of each fraction is expressed as a percentage of the total nitrogen of the protein less the amide nitrogen.

From these figures it will be seen that the amide nitrogen of dephosphorised caseinogen is distinctly lower than that of caseinogen. There is also some decrease in the arginine nitrogen accompanied by an increase in the lysine + cystine fraction of the former. Arginine, in the free state, is known to be attacked by hot sodium hydroxide with the liberation of ornithine and ammonia and it is highly probable that a similar change has here taken place. The resulting ammonia would be evolved along with that derived from the amide nitrogen groups whilst the other product, ornithine, would become included in the figure for lysine + cystine nitrogen.

The nature of the groups giving rise to what is termed amide nitrogen on acid hydrolysis is not known with certainty, although there is evidence in support of the view put forward by Osborne that this nitrogen is present in the protein in the form of amides of the dicarboxylic acids; and in the particular case of caseinogen, Luck [1924] has succeeded in isolating a dipeptide of lysine and glutamine in which this linkage is present. It must be admitted however that the substance isolated by Luck represents only a portion of the groups capable of yielding ammonia when caseinogen is subjected to hydrolysis.

The ease with which a portion of the amide nitrogen fraction of caseinogen is converted into ammonia by the action of relatively weak alkali at 37° is a point of some interest and is in keeping with the supposition that acid amide groups exist in the protein.

It will be seen however that there still remains some 4 % of the total nitrogen of dephosphorised caseinogen in the form of amide nitrogen and in order to ascertain whether this also could be removed by the prolonged action of sodium hydroxide, of the same strength as previously employed, the following experiments were performed in which the rate of ammonia liberation was followed.

Equal volumes of a 4 % solution of caseinogen and of 0.5 *N* sodium hydroxide, both previously warmed to 37°, were mixed and a sample at once taken for total nitrogen determination, the remainder being transferred to a well-stoppered bottle, held in a thermostat at 37° and containing a little paraffin to prevent foaming. A rapid stream of air was then drawn through the solution and the liberated ammonia collected in standard acid, the excess of acid being titrated at suitable intervals and the ammonia determined by difference.

The result of such an experiment is shown in Fig. 1.

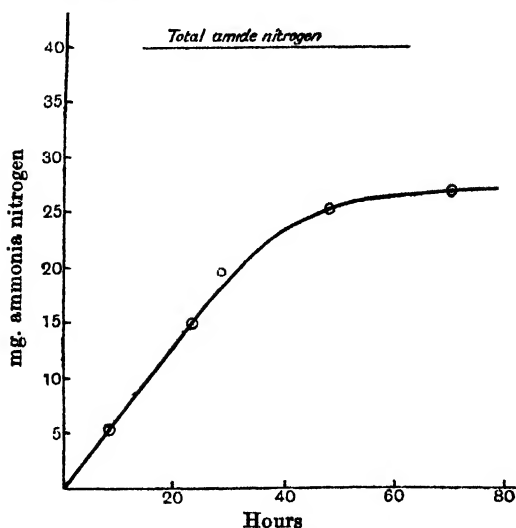


Fig. 1. Liberation of ammonia from caseinogen by 0.25 *N* sodium hydroxide at 37°.

There is a rapid liberation of ammonia during the first 30 hours, at the end of which time approximately 50 % of the total amide nitrogen has been eliminated. During this initial stage the velocity of ammonia production remains constant but subsequently falls. At the end of 70 hours the reaction has almost ceased, there being still however a very gradual liberation of ammonia not amounting to more than 0.5 mg. in 24 hours.

The total ammonia production up to this point is equivalent to about 65 % of the total amide nitrogen. The remainder appears to be comparatively stable towards alkali of the strength employed in these experiments.

Dephosphorised caseinogen gives all the colour tests which are given by caseinogen. It is insoluble in water but soluble in sodium hydroxide, requiring approximately the same quantity of alkali as caseinogen to effect complete solution. It is only very slowly attacked by proteolytic enzymes, a circumstance which may be due to some racemisation having taken place during its preparation by action of the alkali upon the protein. It is not coagulated by rennin.

#### SUMMARY.

1. The low nitrogen content of dephosphorised caseinogen in comparison with caseinogen is accounted for by its much lower content in amide nitrogen.

2. During the action of sodium hydroxide upon caseinogen, ammonia is evolved, derived chiefly from the groups giving rise to amide nitrogen on acid hydrolysis, but also to some extent as a result of the decomposition of arginine.

3. Dephosphorised caseinogen is only slowly attacked by proteolytic enzymes. It is not coagulated by rennin.

The writer desires to record his gratitude to Sir F. G. Hopkins for his continual interest and advice. His thanks are also due to the Department of Scientific and Industrial Research for a grant held throughout the course of this work.

#### REFERENCES.

- Luck (1924). *Biochem. J.* **18**, 679.  
Plimmer and Rosedale (1925). *Biochem. J.* **19**, 1004.  
Rimington and Kay (1926). *Biochem. J.* **20**, 777.

# XXIX. THE EFFECT OF EXCESSIVE RADIATION WITH ULTRA-VIOLET LIGHT UPON THE GROWTH OF RATS.

BY JOAN LEIGH LEIGH-CLARE.

*From the Department of Experimental Pathology, Lister Institute, London.*

*(Received January 1st, 1927.)*

A SERIES of experiments was made in this Institute [Chick and Roscoe, 1926] in which the vitamin A content of milk was titrated by a method based on that of Steenbock, Nelson and Black [1924]. In this method the milk doses were given after growth had ceased upon the defective diet, but the vitamin D requirement of the rat was satisfied by means of daily irradiation with ultra-violet light for 10 minutes or longer. This irradiation was continued during the test period.

It was found that the growth which had been restored by addition of milk to the diet, tended to diminish after 3 to 4 weeks, and more quickly if the time of irradiation was increased from 10 to 20 minutes daily. At the same time the rats developed conjunctivitis with purulent secretion.

An excessive dose of irradiation was clearly deleterious, and it was suggested to me by Dr Chick that it might be worth while to determine whether the effect was entirely due to the conjunctivitis, and also whether it was enhanced when the animals' diet was defective.

The following two experiments were therefore designed to determine:

(1) whether there is a maximum period of time and intensity up to which irradiation with ultra-violet rays is beneficial to growth, and beyond which it is deleterious;

(2) whether the presence or absence of vitamin A in the diet has any influence on the supposed deleterious effects of prolonged exposure to such irradiation.

## TECHNIQUE.

Young rats of 40–50 g. weight received a diet, "F" diet [Chick and Roscoe, 1926], complete in all other respects, but devoid of fat-soluble vitamins. In exp. 1 this was supplemented in some cases by daily doses of pasture-fed butter, to provide vitamin A. In exp. 2 the rats received the same diet without supplement of butter.

The mercury vapour quartz lamp was made by the Hewittic Electric Co., with a straight burner 10 cm. in length; on a 200 volt circuit it was using a current of 4 amperes. It had been in use for about 2 years at the beginning

of the experiment. The rats were exposed at a distance of 42 cm. from the lamp.

*Exp. 1.* The rats were divided into groups receiving irradiation from the lamp for 2', 5', 10' and 30' respectively. Each group contained 6 rats of which 3 received 0.2 g. of butter daily, and 3, 0.5 g. In addition there was a group of 2 rats receiving 1.0 g. of butter and 30' irradiation and control rats which received 0.5 g. of butter and were not irradiated.

The first experiment was begun without arranging for protection to the rats' eyes. After about 12 days, traces of purulent secretion were observed in the eyes of some rats receiving 10 to 30 minutes' irradiation, and steps were at once taken to shield them. This was effected by means of a small circular wash-leather mask with a hole in the centre. The snout was passed through the hole. The mask covered the rats' eyes. A bag of wide-meshed mosquito-net was stitched to the mask and tied firmly behind the head and also behind the fore-legs with strings of narrow tape threaded through the meshes of the mosquito-net. The open end of the bag was secured firmly by safety-pins behind the hind-quarters and this prevented the rats from wriggling out.

With this protection the condition and the eyes improved rapidly and in the course of a week or 10 days the appearance was again normal. Later on, after 1 to 6 weeks, it was noticed that the eyes became sore and showed a blood-stained purulent secretion. The time of onset did not appear to depend upon the length of daily exposure to the ultra-violet radiation; but it was noticed that the rats affected were those whose eyes had been unprotected at the beginning of the experiment, the others continuing to show healthy eyes. This affection of the eyes may have been due to a slightly incomplete supply of vitamin A, but the deficiency must have been very slight, especially in the case of rats receiving the larger doses of butter. The exposure of the eyes at a previous period seemed to have rendered them hyper-sensitive to some slight defect.

It is conceivable that this abnormality may have been concerned with an insufficiency of vitamin B. McCollum, Simmonds and Becker [1925] describe an ophthalmia indistinguishable from that caused by vitamin A deficiency, but which they attribute to an insufficiency of vitamin B, combined with a badly balanced salt mixture in the diet. Hume (verbal communication) has also observed a slight degree of ophthalmia in rats on diets well supplied with vitamin A, which she attributes to an insufficiency of vitamin B.

There was no significant difference to be detected in the first experiment as regards growth in weight between the animals in the different groups, with the exception of the male control which was considerably below the average weight of the irradiated male rats. Disregarding a few exceptions evenly distributed in the various groups, growth was sustained throughout the observation, which lasted 12 to 13 weeks, and there was no failure in the case of rats receiving the largest doses of irradiation. The smallest dose of butter (0.2 g.) appeared to be as effective as the larger doses.



*Exp. 2.* The rats were divided into three groups, one group of 2 rats receiving no irradiation and two groups of 3 rats receiving daily irradiation for 2' and 30' respectively. These rats received no supplement to the "F" diet. The eyes were protected during irradiation of the animal from the beginning of the observation.

As was to be expected, the irradiated rats grew better than the controls but there was no significant difference in this respect between the two groups of irradiated animals. Seven out of the eight rats displayed a more or less marked degree of xerophthalmia, the onset of which was earlier in the majority of the irradiated rats than it was either in their own controls or in the irradiated rats receiving butter (exp. 1). The difference was not however very striking and as the one rat in which the eyes remained normal was an animal receiving the maximum dose of irradiation, the experiment does not seem to suggest that these symptoms of vitamin A deficiency were aggravated by exposure to ultra-violet light.

#### SUMMARY AND CONCLUSIONS.

(1) Previous observations in which ultra-violet radiation had exercised a deleterious effect upon the health of experimental rats are confirmed but the effect is found to be due to the conjunctivitis from which the animals suffered.

(2) Provided that the eyes were shielded, exposure of young growing rats to ultra-violet radiation for periods up to 30' daily was found to have no deleterious effect upon the growth and well-being of the rats (although this dose was found to be 15 times the adequate dose for male rats). It was also found that exposure for 30' to the lamp had no more beneficial results than exposure for the shorter periods.

(3) When using the biological method for the titration of vitamin A it is preferable to provide vitamin D by the inclusion of an irradiated vitamin-free rat in the diet, than by direct irradiation of the test rats.

Thanks are due to Dr H. Chick, Prof. C. J. Martin and Miss E. M. Hume, for kind help and criticism, and to the Lister Institute for permission to carry out the experiments there.

#### REFERENCES.

- Chick and Roscoe (1926). *Biochem. J.* **20**, 633.  
McCollum, Simmonds and Becker (1925). *J. Biol. Chem.* **64**, 161.  
Steenbock, Nelson and Black (1924). *J. Biol. Chem.* **59**, *Proc.* ix.

# XXX. A FURTHER NOTE ON THE ANTIRACHITIC VALUE OF FRESH SPINACH.

By MARGARET HONORA ROSCOE.

*From the Department of Experimental Pathology, Lister Institute, London.*

*(Received January 3rd, 1927.)*

THE presence of the antirachitic vitamin D in green leaves has been a matter of considerable uncertainty. McClendon and Shuck [1923], and Zucker and Barnett [1923], in dietetic experiments with rats receiving low phosphorus rachitic diets, could not detect any antirachitic properties in spinach leaves.

On the other hand, cut green leaves irradiated by the mercury vapour lamp have been shown to acquire active antirachitic properties [Hess and Weinstock, 1924, 1925; Chick and Roscoe, 1926, 1], and it seemed probable that the same synthesis would take place to some extent in leaves exposed to the ultra-violet rays in the sunlight. This has however never been conclusively proved.

Chick and Roscoe [1926, 1] showed that additions of fresh, summer-grown spinach to a diet adequate in calcium and phosphorus produced a slight improvement in calcification of the bones of young rats. No improvement was obtained with spinach grown in the winter. The observations of Luce [1924], and Chick and Roscoe [1926, 2], working with a milch-cow, gave some indication that the antirachitic value of the milk was slightly higher when the cow, itself exposed to summer sunshine, was being fed on green fodder than when it was fed on dry food.

Chick and Roscoe [1926, 1] have put forward two theories to account for the discrepancy between the antirachitic value of spinach exposed to the ordinary sunshine and that irradiated by ultra-violet light from an artificial source. (i) That the antirachitic vitamin is present in such small amounts that the effect could not be demonstrated with the quantities hitherto employed. (ii) That though the vitamin is synthesised in the green leaves it is rapidly removed or destroyed.

To examine the latter hypothesis it was decided to supplement the vitamin D-deficient diet of a set of rats with spinach picked in the open at mid-day and immediately fed to them.

To obviate the difficulty described under (i), rabbits were used in one experiment, since they will eat a much larger amount of green stuff in relation to the basal diet than will rats.

## EXPERIMENTAL.

McCollum's No. 3143 low phosphorus, rickets-producing diet was used [McCollum, Simmonds, Shipley and Park, 1921]. With young rats on this diet a greater difference in bone calcification was found between those animals receiving the basal diet alone and those receiving the basal diet, supplemented with abundant vitamin D, given as cod-liver oil or by irradiation, than when a diet adequate in phosphorus was used. A better histological picture of rickets was also found.

The rabbits had the same diet, the only difference being that it was given as a dry mixture of ground cereals, gelatin and salts, etc. Water was given separately. It was eaten well, except that some of the gelatin was always left. Both control rabbits fed on this diet showed definite rickets.

The spinach given was of the prickly seeded variety (*Spinacea oleracea*). That given to the rats was grown in boxes on the roof of this Institute, during the months of June and July, 1926. It was picked and fed to the animals at mid-day, having been in what sunlight there was all the morning. There were not many days during the period without some sunshine. As this source was not adequate to supply the rabbits, they received bought spinach in addition. In some cases this was exposed to the sunlight, with the cut ends in water, for 3 to 4 hours before feeding ("sunned spinach"); in others it was fed as bought from the shop.

The largest average amount of spinach eaten per rat per day was 1.5 g. These rats were eating 15 g. of food daily. The dose therefore formed 10 % of the wet weight of the diet. The rabbits ate 40 g. of the spinach and about 40 g. of dry basal diet (equivalent to 160 g. of wet diet) daily. The spinach therefore formed 25 % of the wet weight of their diet. The rabbits would have eaten more spinach, but it was thought inadvisable to alter the ratio of calcium to phosphorus in the food to any greater extent; by the 25 % dose of spinach it was lowered from 4.1 : 1.0 in the basal diet, to 3.8 : 1.0 in the diet with spinach addition. This alteration cannot have been large enough to account for the results obtained [McCollum *et al.*, 1921].

*Exp. 1. Young rats.* Five male rats from one litter, approximately 40 g. in weight, were fed on McCollum's 3143 diet. One received the basal diet alone, three were given in addition as much spinach as they would eat, and the fifth was given cod-liver oil. At the end of four weeks they were killed, their leg bones analysed by the method described by Chick, Korenchevsky and Roscoe [1926], and a histological examination of their rib junctions made. The results are set out below.

	Ca % on fat-extracted dry weight of leg bones*	% increase in calcification	A/R ratio
Control	8.61	—	0.303
Spinach average	9.86	11	0.363
per diem	9.06	10	0.348
1.5 g.	9.56	11	0.332
Cod-liver oil 0.05 g.	15.27	77	0.703
per diem			
	average = 9.49	average = 11	average = 0.348

\* Calculated as 37 % of the ash.

All the rats, except the one receiving cod-liver oil, exhibited angulation of the rib junctions and fractures, but this was more severe in the negative controls than in the others.

The A/R ratio, or the ratio of the ash of the bones to the organic residue of the fat-extracted bone, is included as giving a very satisfactory criterion of bone calcification in young rats [Chick, Korenchevsky and Roscoe, 1926].

The proportion of calcium in the leg bones, reckoned on the fat-extracted weight, is increased from 8.6 % to 9.5 % or by about 11 %. This increased calcification is however far short of that achieved in the "positive control animal" which received 50 mg. of cod-liver oil per day and showed 15.3 % calcium in the fat-extracted bones. The A/R ratio is increased from 0.303 to 0.318.

A definite slight increase in calcification is noticeable. The histology shows severe rickets in all rats except the one receiving cod-liver oil, which was normal.

*Exp. 2. Young rabbits.* Six young rabbits of one litter were placed on the basal diet at the age of 4 weeks, when weighing approximately 300 g. each. Four of these received a supplement of fresh spinach leaves, in two cases as it was bought from the shop, in two after it had been exposed to sunshine. At the end of 5 weeks they were killed, the femurs of both legs analysed and the rib junctions examined histologically. The results are set out below.

	Ca % on fat extracted dry wt. of femur*		% increase in calcification		A/R ratio		Histology	
	1	2	3	4	5	6	7	8
Controls	15.78	19.15	—	—	0.745	1.029	Definite rickets	Slight rickets
Spinach 40 g. per diem (as bought)	17.12	18.64	11	3	0.845	1.068	Slight rickets	Very slight rickets
Spinach 40 g. per diem (sunned)	16.84	20.73	11	11	0.826	1.226	Slight rickets	Normal

The control animals exhibited macroscopic rickets with swollen rib junctions; the ribs of the male control had also several fractures.

If the rabbits receiving spinach be compared only with the controls of their own sex, it will be seen that in three out of the four cases the percentage calcification of their bones is raised, that the calcification as measured by the A/R ratio is raised in all cases, and that the histology is also improved in all cases. The animals receiving sunned spinach do not show a consistent improvement over those receiving the unsunned, either in calcification or in histology.

The calcification in the males is consistently lower than in the females and the histological rickets more severe. This, if always so, constitutes a serious difficulty in the setting out of experiments with young rabbits as it is practically impossible to determine the sex at four weeks.

\* The Ca was determined directly by a modification of McCrudden's method [Boas, 1924]. It was found to be equal to 37 % of the ash in rabbits as in rats.

## DISCUSSION.

All but one of the animals receiving spinach showed a slight increase in the calcium content of the bones compared with the control animals. Taking the A/R ratio as the criterion of calcification the increase is consistent. This confirms the previous results of Chick and Roscoe [1926, 1] with summer-grown spinach.

No greater percentage increase in the calcium content of the bones was observable in the rabbits, 25 % of whose diet was spinach, than in the rats, 10 % of whose diet was spinach. This may be due to differing requirements of rats and rabbits for vitamin D. At the same time it may be noted that the calcium content of the bones of the rat which received 1.5 g. of spinach was no greater than that of the one which received 0.8 g.

Histologically, whereas the rats all showed rickets to such a marked degree that no distinction could be made between the controls and those receiving spinach, the rabbits receiving spinach showed an improvement from pronounced to slight rickets in the males, and from slight rickets to normal in the females.

The presence of vitamin D in small amounts in fresh spinach is therefore demonstrated. Improvement in the histology is shown better in experiments with rabbits than with rats. This is probably due to the larger amount of spinach eaten.

These experiments do not afford any evidence in favour of the theory that vitamin D, after synthesis in the leaf, is rapidly removed. In Exp. 2, carried out with spinach leaves which, with the cut ends in water, were placed in the sun immediately before being given to the animals, the results were no better than when the spinach supplement was not so treated. In Exp. 1, carried out with leaves which were on the plant in the sun till the time of feeding, calcifying power was demonstrated, but this was no greater than that obtained by Chick and Roscoe [1926, 1] using the same quantities of leaves picked in the evening of the day before they were fed.

## SUMMARY.

1. A slight but definite influence upon calcification is again demonstrated by adding fresh green leaves of summer spinach to the diet of young rats and rabbits.
2. This effect is more obvious in experiments with rabbits than with rats.
3. The difference of opinion as to the presence of vitamin D in green leaves is probably due to the difficulty of demonstrating the small amounts contained.

My thanks are due to Prof. C. J. Martin and to Dr H. Chick for their advice and criticism during this research, and to Prof. Korenchevsky for help with the histology.

## REFERENCES.

- Boas (1924). *Biochem. J.* **18**, 425.  
Chick, Korenchevsky and Roscoe (1926). *Biochem. J.* **20**, 622.  
Chick and Roscoe (1926, 1). *Biochem. J.* **20**, 137.  
—— — (1926, 2). *Biochem. J.* **20**, 632.  
Hess and Weinstock (1924). *J. Biol. Chem.* **62**, 301.  
—— — (1925). *J. Biol. Chem.* **63**, 297.  
Lucas (1924). *Biochem. J.* **18**, 716, 1297.  
McClendon and Shuck (1923). *Proc. Soc. Exp. Biol. Med.* **20**, 288.  
McCollum, Simmonds, Shipley and Park (1921). *J. Biol. Chem.* **47**, 507.  
Zucker and Barnett (1923). *Proc. Soc. Exp. Biol. Med.* **29**, 375.

# XXXI. OPTICAL ROTATORY POWER AND DISPERSION OF PROTEINS.

By LESLIE FRANK HEWITT.

*From the Hale and Dunn Clinical Laboratories, London Hospital, E 1.*

*(Received January 1st, 1927.)*

THE importance of optical rotatory power for the characterisation of proteins has received attention since 1880 [Fredericq, 1880; Starke, 1881; Sebilin, 1885].

There is however a wide divergence in the results obtained; *e.g.* in the case of serum albumin values for the specific rotatory power for the D-line vary from  $-33^{\circ}$  [Reiss, 1904] to  $-63^{\circ}$  [Young, 1922]; other values being  $-47.8^{\circ}$  [Maximovitsch, 1901],  $-42^{\circ}$  to  $-49^{\circ}$  [Gürber, 1894],  $-18^{\circ}$  [Michel, 1895],  $-51^{\circ}$  to  $-66^{\circ}$  [Lewis, 1922]. Similarly in the case of serum globulins, values varying from  $-42^{\circ}$  [Porges and Spiro, 1902] to  $-51.2^{\circ}$  [Seng, 1899] and  $-52^{\circ}$  [Lewis, 1922] have been recorded. This divergence alone renders it desirable that the data be critically examined and certain of the determinations repeated. Also, until a recent publication of Hafner [1925] which appeared after the present work had commenced, no attention has been paid to the rotatory power of the proteins for light of different wave-lengths, *i.e.* their optical rotatory dispersion.

From many points of view we consider this work of Hafner to be unsatisfactory. Like all other workers in this field except Hardy and Gardiner [1910] and Young [1922] lipins were not removed from the proteins although these considerably modify their properties. In addition Hafner made no attempt to separate euglobulin and pseudoglobulin although they have different rotations: and the use of very dilute solutions for optical measurements and the lack of duplicate determinations make the results even less dependable. As will be shown later, the results are not acceptable on other experimental and theoretical grounds.

The optical rotation and dispersion of lipin-free serum and other proteins have therefore been determined.

## EXPERIMENTAL METHODS.

### *Serum proteins.*

Lipins were extracted from serum by a modification of Hardy and Gardiner's method [1910] and that of Hartley [1925]. Plasma, or preferably serum (100 cc.), at  $0^{\circ}$  is poured into a mixture (600 cc.) of alcohol (7 parts) and ether (3 parts) cooled to  $-15^{\circ}$ . The mixture is vigorously stirred and the

temperature not allowed to rise above  $-5^{\circ}$ . After allowing to stand at this temperature in an ice-chest for 2 hours the precipitated proteins are filtered off on a Büchner funnel in the ice-chest, washed several times with portions (40 cc.) of the alcohol-ether mixture at  $0^{\circ}$ , and with several portions (50 cc.) of ether at  $0^{\circ}$ . The filtration accelerates markedly when the ether washing is commenced. The proteins are then rapidly transferred to extraction thimbles and extracted for 2 hours in a Soxhlet apparatus with ether, metallic sodium being placed in the extraction flask to remove alcohol extracted from the proteins. After 2 hours' extraction the ether and sodium in the apparatus are renewed and the extraction is continued for 20 hours. It is found that if the proteins are dried *in vacuo* before ether extraction, an appreciable amount of alcohol remains in the precipitate and considerable denaturation occurs. After completion of the ether extraction the proteins are transferred to a desiccator and dried *in vacuo* for 48 hours. The dried proteins are not denatured by standing in contact with a trace of ether as they are by alcohol. When freed from ether the proteins are allowed to stand for a while in contact with laboratory air to become slightly moist, otherwise, when completely dry, they are difficult to work with, easily electrified and adhere to surfaces. Obtained in this manner the extracted proteins are white, powdery solids readily dissolving in water giving clear solutions. Reconstituted plasma can still be caused to clot and albumins may be crystallised.

The extracted serum proteins are dissolved in water and the volume made up to that of the original serum, and the englobulin and pseudoglobulin removed in turn by additions of saturated ammonium sulphate solution to render the mixture one-third and one-half saturated with ammonium sulphate. The albumins are then either caused to crystallise or precipitated by addition of small amounts of 10 % acetic acid and saturated ammonium sulphate solution, the reaction being adjusted to  $p_H$  about 4.9. Precipitation of albumin by adjusting the reaction and addition of ammonium sulphate solution is preferable to addition of solid ammonium sulphate. The protein fractions are filtered off on Büchner funnels using Schleicher and Schüll hardened papers (No. 575), redissolved in water and reprecipitated twice more in a similar manner. This fractionation is sufficient for the present purpose. The proteins are then dissolved in water and dialysed in collodion membranes against distilled water in an ice-chest, the outer liquid being changed hourly for the first day and then daily for at least a week. The membranes used were tested for permeability to salts and dyes and were all impermeable to serum proteins. In the case of albumin and pseudoglobulin the dialysed solutions were used directly for the determination of rotatory power whilst the euglobulin was precipitated as a white solid on dialysis and was dissolved in 0.9 % sodium chloride solution. The solutions used were clear and colourless except the albumins which have a slight yellow colour in thick layers.

A Schmidt and Haentzsch polarimeter with a Hilger direct vision spectroscopic eyepiece was used for the measurement of rotations and a mercury



vapour lamp provided a light source for wave-lengths, 4358 (blue), 5461 (green), and 5780 (yellow) Ångström units, and a Nernst lamp with a crystal-violet filter, red light (6600 Å.u.). For the determination of proteins the author is indebted to Dr J. R. Marrack who used the micro-Kjeldahl method, using the factor 6.45 for the conversion of nitrogen content to protein concentration.

*Serum albumin.*

In this section all rotatory powers are given for the mercury green line at 20° ( $[\alpha]_{5461}^{20^\circ}$ ), rotations for this light being most accurately determined.

Table I.  $[\alpha]_{5461}^{20^\circ}$  of serum albumins.

	Crystalline			Precipitated				Mean
	Horse 1	Horse 2	Horse 3	Horse 4	Ox 5	Human 6	Human 7	
$p_H$ 4.9	-78.4°	-81.0°	-78.7°	-76°	-79.6°	-79.3°	-77.7°	-78.1°
$p_H$ 7.2	-78.4°	-81.5°	-81.8°	—	—	—	—	—

It is to be noted that:

(1) Precipitated and crystallised extracted serum albumins have approximately equal rotatory powers.

(2) Alteration of the  $p_H$  from 4.9 to 7.2 has but little effect on the rotation.

(3) Horse, ox, and human serum albumins have about the same rotatory powers.

(4) The results are in agreement with those of Young [1922], *i.e.* -78.3° in place of -78.1°, in contradistinction to those of Lewis [1922] and other workers who worked with proteins contaminated with lipins. Lewis used only the D-line for his determinations of rotatory power, but, on extrapolating his results on the basis of the curves obtained by us, his value for horse albumin is -70°. Using unextracted serum albumin we have obtained similar results, but, as Lewis found, it is difficult to obtain concordant results with such material.

If the albumins are allowed to remain in contact with alcohol at the ordinary temperature, or with acidified ammonium sulphate solution for long periods, or if the protein solutions are heated, a certain amount of denaturation occurs and the rotatory power is increased. Samples 8, 9 and 10 in Table II and possibly 2 in Table I were partly denatured.

Table II. *Denatured albumins (horse serum).*

	8	9	10
	Crystalline	Precipitated	
$[\alpha]_{5461}^{20^\circ}$	-90.0°	-89.0°	-84.3°

*Serum pseudoglobulin.*

Table III.  $[\alpha]_{5461}^{20^\circ}$  of serum pseudoglobulin in water.

Horse 1	Horse 2	Horse 3	Human 4	Ox 5	Mean value
-68.8°	-66.2°	-69°	-70.7°	-69°	-68.7°

Good agreement is obtained between the values of  $[\alpha]_{5461}^{20^\circ}$  obtained for extracted horse, human and ox serum pseudoglobulin. The mean value of  $-68.7^\circ$  is higher than that calculated from Lewis's results for unextracted horse serum pseudoglobulin ( $-62.1^\circ$ ).

*Serum euglobulin.*

Table IV.  $[\alpha]_{5461}^{20^\circ}$  of extracted serum euglobulin.

Horse		Ox	Mean
1	2	3	
$-60.1^\circ$	$-61.3^\circ$	$-62.1^\circ$	$-61.2^\circ$

Good agreement is obtained between the values of  $[\alpha]_{5461}^{20^\circ}$  for horse and ox extracted serum euglobulin and the values are higher than those calculated from Lewis's figure ( $-51.0^\circ$ ) for unextracted horse euglobulin.

*Egg-albumin.*

To the whipped white of fresh eggs is added an equal volume of saturated ammonium sulphate, and after standing over night the precipitate is filtered off and the albumin in the filtrate precipitated by acidification and addition of ammonium sulphate. The precipitated albumins are filtered off, dissolved in water and extracted first with alcohol and ether in the cold, and then with ether at the ordinary temperature, and then dried, as described in the case of serum proteins. The extracted egg-albumin thus obtained gives a clear solution in water even at the isoelectric point and crystallises, but with some difficulty, in the ordinary way. Sørensen [1926] expresses the opinion that egg-albumin cannot be extracted with alcohol and ether in the cold without denaturation, and takes as criteria of undenatured egg-albumin, solubility in water and crystallisability. The extracted protein is dissolved in water, the solution made neutral, an equal volume of saturated ammonium sulphate is added, the solution filtered and the egg-albumin twice precipitated by additions of appropriate amounts of 10 % acid and saturated ammonium sulphate solution. The egg-albumin thus obtained is dissolved in water and dialysed until free from salts in the usual way. The albumin contained 7.7 mg. of phosphorus per 1 g. of nitrogen which corresponds to a molecular weight for egg-albumin of about 26,000, assuming there is 1 atom of phosphorus per molecule of protein. The generally accepted values for the molecular weight lie between 27,000 and 34,000 [cf. Cohn, Prentiss and Hendry, 1925]. The specific rotatory power,  $[\alpha]_{5461}^{20^\circ} - 44.5^\circ$ , is higher than that obtained by Young [1922] for unextracted egg-albumin ( $-37.5^\circ$ ).

*Lactalbumin.*

The caseinogen is removed from fresh skimmed milk either by addition of dilute acetic acid under the surface with vigorous stirring or by treatment at  $37^\circ$  with rennin for 45 minutes. The precipitated casein is removed by

filtration, and the lactalbumin is precipitated by addition of dilute acetic acid until the solution has  $p_H$  about 4.9, and of saturated ammonium sulphate solution. After standing, the precipitated proteins are filtered off, dissolved in water and extracted with a mixture of alcohol and ether in the cold and with ether in a Soxhlet apparatus, and dried *in vacuo* in the usual way. The white powdery protein is dissolved in water, the solution made neutral, an equal volume of saturated ammonium sulphate added, and the solution filtered. The lactalbumin is then twice precipitated by addition of dilute acetic acid and saturated ammonium sulphate solution, and filtered on Büchner funnels. The purified lactalbumin is then dissolved in water and dialysed until free from salts. The rotatory power is  $[\alpha]_{5461}^{20} - 47.5^\circ$ . The phosphorus content is 5.4 mg. per 1 g. of nitrogen in the protein. The molecular weight of lactalbumin, assuming that there is 1 atom of phosphorus in each molecule of lactalbumin, is thus about 37,000. For the phosphorus determinations the author is indebted to Dr Kay.

#### Caseinogen.

Caseinogen was prepared from milk by the method of Van Slyke and Baker [1918] as modified by Cohn [1922].

#### Gelatin.

Isoelectric gelatin was prepared from Coignet's "Gold Label" gelatin by the method of Loeb [1922].

Table V. *Specific rotatory powers of proteins and tyrosine for light of different wave-lengths.*

	$[\alpha]_D^{20}$			
Protein	$\lambda$ 4359	$\lambda$ 5461	$\lambda$ 5780	$\lambda$ 6600 Å.U.
Serum albumin	-151.5'	-78.1°	67.0	47.3
Serum pseudoglobulin	118.2	-68.7	-59.8	-43.1
Serum euglobulin	105.2	-61.2	-54.0	39.4
Egg-albumin	-83.9	44.5	-38.3	27.5
Lactalbumin	-88.9	-47.5	-41.1	-28.6
Caseinogen	-184.4	-105.1	-91.2	66.0
Gelatin	-496.8	-282.8	-248.2	181.1
Tyrosine in 20 % HCl	-7.8	-9.6	-9.3	-7.2
" 4 % HCl	14.4	12.5	-11.5	8.9
" 1 % NaOH	-22.9	-15.1	-13.1	-9.8
" 20 % NaOH	27.8	-17.4	-15.2	-11.2

In Table V are given the specific rotatory powers of the proteins examined for light of different wave-lengths. To confine attention for the moment to the rotation for the mercury green line ( $\lambda = 5461$  Å.U.), which is probably most accurately determined, the following conclusions may be drawn.

(i) The considerably greater (10-15 %) rotation of lipin-free proteins, as pointed out in the experimental section.

(ii) The marked difference between the rotation of serum albumin and globulins, and between serum pseudoglobulin and euglobulin.

(iii) Caseinogen has a high laevo-rotation,  $[\alpha]_{5461}^{20} = -105^\circ$ , not a dextro-rotation as is inferred from the work of Zaykowsky [1923]. Tyrosine is included to show the effect of  $p_H$  on dispersion. This will be referred to later.

#### OPTICAL ROTATORY DISPERSION.

It is well known that, in general, optical rotation increases with decreasing wave-length of the light used for the determination; but Drude in a treatise on optics in 1907 was the first to obtain a satisfactory mathematical expression connecting rotatory power and wave-length of the light.

Drude's equation is:

$$\alpha_\lambda = \frac{k_0}{\lambda^2 - \lambda_0^2} \pm \frac{k_1}{\lambda^2 - \lambda_1^2} \dots \text{etc.},$$

where  $\alpha_\lambda$  = rotation for light of wave-length  $\lambda$ ,

$k_0, k_1$  = constants,

$\lambda_0, \lambda_1$  = constants.

The rotation may be controlled by one, two, or more terms in the Drude equation.  $\lambda_0, \lambda_1$  etc., have a further significance in that they are the characteristic wave-lengths of oscillating electrons in the molecule and as such give rise to bands in the absorption spectrum of the substance, so that a direct relationship exists between the rotatory dispersion and the absorption spectrum of the substance. Lowry [1913] was among the first to apply the Drude equation to chemical substances. He showed that if the rotatory powers of a substance can be expressed, as those of most substances can, by a single term Drude equation, i.e.  $\alpha_\lambda = \frac{k_0}{\lambda^2 - \lambda_0^2}$ , then a linear relationship exists between the reciprocals of the rotatory powers  $\left(\frac{1}{\alpha_\lambda}\right)$  and the squares of the wave-lengths ( $\lambda^2$ ). This follows from a rearrangement of the equation:

$$\frac{1}{\alpha_\lambda} = \frac{\lambda^2 - \lambda_0^2}{k_0};$$

and if  $\frac{1}{\alpha_\lambda} = 0$ , then  $\lambda = \lambda_0$ . That is to say, if  $\frac{1}{\alpha_\lambda}$  is plotted against  $\lambda^2$  the point where the line cuts the zero axis will indicate the wave-length of the absorption band controlling the dispersion.

In Fig. 1 are plotted the dispersions of the proteins in the way described. No deviation from a linear relationship between  $\frac{1}{\alpha_\lambda}$  and  $\lambda^2$  in the visible region of the spectrum can be detected.

Serum albumin, egg-albumin and lactalbumin all have similar dispersions, the dispersion curve cutting the axis at a point at, or near to,  $\lambda = 2760 \text{ \AA.}$ , which is the wave-length of the head of the absorption band in serum proteins nearest to the visible portion of the spectrum. In serum pseudoglobulin, euglobulin and caseinogen, however, the line appears to strike the axis at a point nearer the region of shorter wave-length, although these proteins have similar absorption spectra to the albumins. It would thus seem that in the albumins the absorption band of longest wave-length dominates the dispersion, whilst in the other proteins other bands have a predominant effect.

The case of gelatin is interesting since, owing to its low content of amino-acids containing a benzene nucleus, it does not possess absorption bands as near the visible region of the spectrum as the other proteins, and it is found, as expected, that the dispersion is correspondingly modified.

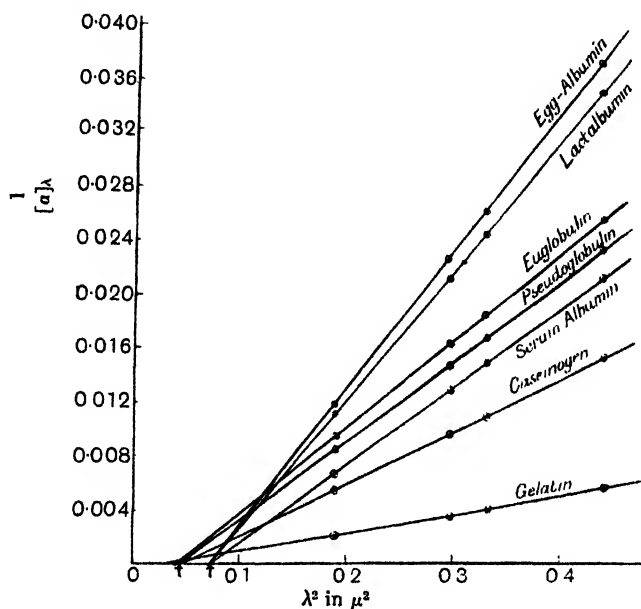


Fig. 1. Dispersion curves of proteins. Relation between  $\frac{1}{[\alpha]\lambda}$  and  $\lambda^2$ .

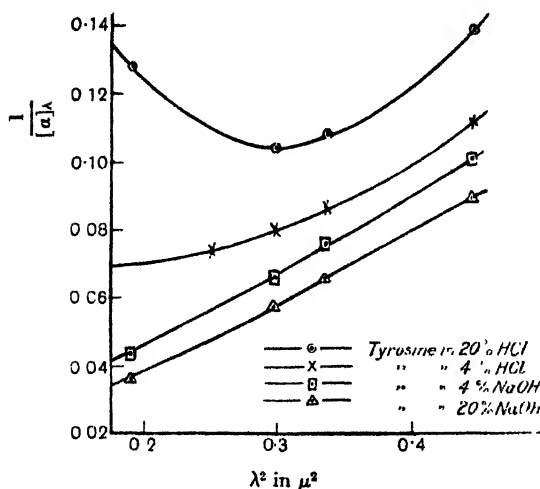


Fig. 2. Rotatory dispersion of tyrosine at different  $pH$ .

The optical rotatory dispersion of tyrosine is more complex than that of the proteins, that is to say, two or more terms of the Drude equation are required to express the rotations for different wave-lengths (see Fig. 2).

Hydrogen ion concentration has a marked effect on the dispersion. In tyrosine therefore several absorption bands have a great influence on the rotation. Tyrosine has a very similar absorption spectrum to that of serum proteins [Stenström and Reinhard, 1925], and it does not vary greatly with alteration of  $p_H$ . In two respects therefore the absorption spectra and rotatory dispersion vary. The absorption spectra of serum proteins and of tyrosine are very similar but the rotatory dispersion is different; and the absorption spectrum of tyrosine is not greatly affected by hydrogen ion concentration whilst the rotatory dispersion is altered by change of  $p_H$ .

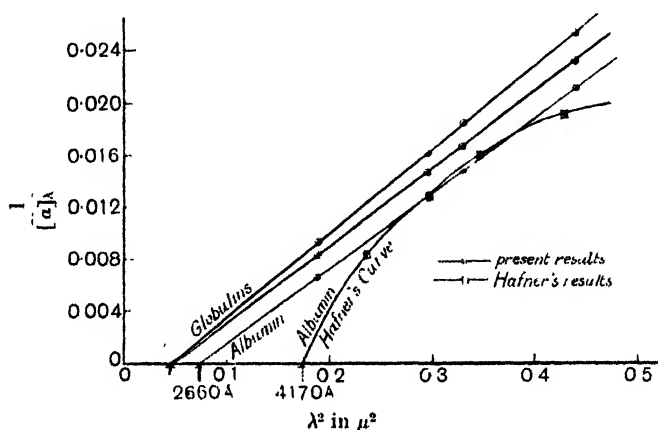


Fig. 3. Dispersion of serum proteins showing Hafner's [1925] results.

Hafner's results [1925] differ markedly from ours. First he finds the rotations of globulins higher than those of albumins, which is not supported by the work of other authors. In Fig. 3 are plotted Hafner's dispersion of serum albumin and our results on serum proteins. Hafner's figures (which are plotted inaccurately in his paper) lead him to the conclusion that the characteristic wave-length of an oscillating electron in the albumin molecule (*i.e.* that of an absorption band) is  $4170 \text{ \AA.U.}$ ; this would involve an absorption band in the visible region of the spectrum, an entirely unconfirmed hypothesis. Also, by carrying the determinations of rotatory power further into the blue region of the spectrum, we have shown Hafner's results to be inaccurate. Deviations of Hafner's curve from the straight line obtained by us are readily explicable on the basis of experimental errors, due to lack of duplicate determinations, dilution of solutions, etc. on Hafner's part.

#### PHYSICAL PROPERTIES OF THE GLOBULINS.

Chick [1914] and other workers express the opinion that euglobulin is a mechanical complex of pseudoglobulin and lipins which can be dissociated by extraction of the lipins with alcohol and ether. We have been unable to obtain evidence of such separation. After extraction with alcohol and ether

at  $-4^{\circ}$  and with ether at laboratory temperature for 24 hours, euglobulin and pseudoglobulin retain their characteristic individual properties.

	Extracted euglobulin	Extracted pseudoglobulin
Solubility in saline	Soluble	Soluble
Solubility in distilled water	Insoluble	Soluble
When dialysed against distilled water	Precipitated	Remains in solution
$[\alpha]_{5461}^{20}$	$-61.2^{\circ}$	$-68.7^{\circ}$

#### SUMMARY.

1. The rotatory powers of lipin-free serum albumin, pseudoglobulin and euglobulin, lactalbumin, egg-albumin, caseinogen and gelatin for different wave-lengths have been determined.
2. Extracted serum albumin, pseudoglobulin and euglobulin have different and distinct rotatory powers. Euglobulin is not a mechanical complex of pseudoglobulin and lipin.
3. Serum albumin, egg-albumin and lactalbumin have rotatory dispersions dominated by an absorption band at about 2760 Angström units. Serum globulins, caseinogen, and gelatin have different rotatory dispersions from the albumins.
4. Extraction of lipins from proteins results in a considerable increase of rotatory power, but crystallised and non-crystalline serum albumins have practically identical properties.

The author wishes to thank Dr J. R. Marrack for his active interest in this work which was carried out under the auspices of the Freedom Research Fund.

#### REFERENCES.

- Chick (1914). *Biochem. J.* **8**, 404.  
 Cohn (1922). *J. Gen. Physiol.* **4**, 697.  
 Cohn, Prentiss and Hendry (1925). *J. Biol. Chem.* **63**, 720.  
 Fredericq (1880). *Arch. Biol.* **1**, 47.  
 Gürber (1894). *Sitz. Würzburger physiol.-med. Ges.* **143**.  
 Hafner (1925). *Biochem. Z.* **166**, 424.  
 Hardy and Gardiner (1910). *J. Physiol.* **40**; *Proc.* **lxviii**.  
 Hartley (1925). *Brit. J. Exp. Path.* **6**, 180.  
 Lewis (1922). *Proc. Roy. Soc. Lond. B*, **93**, 178.  
 Loeb (1922). *Proteins and Theory of Colloidal Behaviour* (New York and London), p. 35.  
 Lowry (1913). *J. Chem. Soc.* **103**, 1062.  
 Maximovitch (1901). *Maly's Jahresber.* **31**, 35.  
 Michel (1895). *Sitz. Würzburger physiol.-med. Ges.* **29**, 117.  
 Porges and Spiro (1902). *Hofmeister's Beiträge*, **3**, 277.  
 Reiss (1904). *Hofmeister's Beiträge*, **4**, 150.  
 Sehilin (1885). *Z. physiol. Chem.* **9**, 445.  
 Seng (1899). *Z. Hyg. Infektionskrank.* **31**, 513.  
 Van Slyke and Baker (1918). *J. Biol. Chem.* **35**, 127.  
 Sørensen (1926). *J. Chem. Soc.* 3007.  
 Starke (1881). *Maly's Jahresber.* **11**, 17.  
 Stenström and Reinhard (1925). *J. Phys. Chem.* **29**, 1477.  
 Young (1922). *Proc. Roy. Soc. Lond. B*, **93**, 15.  
 Zaykowsky (1923). *Biochem. Z.* **137**, 502.

## XXXII. THE ETHER-SOLUBLE SUBSTANCES OF CABBAGE LEAF CYTOPLASM.

### I. PREPARATION AND GENERAL CHARACTERS.

By ALBERT CHARLES CHIBNALL  
AND HAROLD JOHN CHANNON (*Beit Memorial Research Fellow*).

*From the Department of Physiology and Biochemistry,  
University College, London.*

*(Received January 3rd, 1927.)*

IN a previous paper [Chibnall and Grover, 1926], there has been described the preparation in bulk of the cytoplasm from green leaves; in that paper it was shown that the cytoplasm consists chiefly of proteins and substances soluble in fat solvents and the methods of preparation and the properties of these proteins were discussed. As part of this cytoplasmic protein appeared to be in some state of physical or chemical combination with substances of fatty nature, it was deemed wise to attempt to study the nature of the ether-soluble substances of cytoplasm before embarking on more advanced studies of the proteins themselves.

A review of the existing literature at once discloses the rather surprising fact that although numerous references to the isolation of individual substances of fatty nature abound, no thorough investigation of the fatty materials present in the leaf has been attempted. This is probably due to the fact that the extraction of the fatty material from fresh leaves—in which both the cell wall and cytoplasm are heavily imbibed with water—is attended with considerable difficulties. In such work on the plant as has been reported, the seed or young seedlings appear to have been studied chiefly, and the method usually adopted has been to extract with boiling alcohol, evaporate the extract to dryness and to treat the residue obtained with ether. This method cannot be satisfactory when applied to fresh leaf material in bulk. In the first place, the extraction of say 20 kg. of fresh leaves would require a prohibitive amount of alcohol; secondly, the dilution of the alcohol by the water in the leaf is sufficient to cause the solution of a certain amount of the sugars, water-soluble nitrogenous substances and inorganic salts which have to be subsequently removed; thirdly, substances insoluble in alcohol but soluble in ether may be present, and this entails subsequent exhaustive extraction of the bulky leaf material with ether.

Alternatively the leaf material has been dried and ground to a very fine



powder. Although this process results in a much less bulky product and one which can be directly treated with fat solvents, there are objections to it, for the drying of large quantities of leaf material is necessarily slow, with the resulting danger of considerable alteration of the substances present due to enzyme action to an extent which we have no means of judging. Further the continual grinding, which must be carried out if the material is to be reduced to that very fine state of division which is so necessary for successful extraction, may result in a considerable degree of oxidation.

Even if the difficulties of dealing with moderately large quantities of material are overcome, there still remains the fact that the yield of fatty substances from green leaves is, in general, very small in terms of the weight of the fresh leaf taken, even though the fat constitutes no inconsiderable proportion of the total leaf solids. Thus during the investigation reported in this paper, upwards of 200 kg. of fresh leaves have been used, and yet as will be seen later, we have been limited throughout the work for want of sufficient fatty material. These considerations are probably sufficient to explain the paucity of information available on the chemistry of the leaf fats.

The problem of obtaining sufficient material is thus one of devising means whereby very large quantities of leaf material can be reduced rapidly to small bulk in a form which can be directly extracted with fat solvents without the necessity of preliminary drying.

#### GENERAL EXPERIMENTAL METHODS.

The present paper deals not with the ether-soluble substances of the total leaf but only with those which appear to be part of the colloidal complex which constitutes the cytoplasm of the leaf cells. In previous papers methods of obtaining this cytoplasm free from cell-wall material, and the preparation of proteins from it have been described [Chibnall and Grover, 1926]. In those papers it was considered justifiable to give to these proteins the generic name of cytoplasmic proteins. This terminology neglects the nucleus. However, in the mature leaf-cell the mass of the nucleus is very small compared to that of the cytoplasm. Therefore it may be well if we emphasise here the fact that the proteins were obtained in such quantity as to make it quite definite that they must be derived in bulk from the cytoplasm itself and not from the nucleus. Similarly in the present work we have found no single ether-soluble substance which can definitely be assigned to the nucleus, for the methods available for the isolation of the individual constituents of any complex fatty material are such that unless a very large quantity can be used it is a matter of considerable difficulty to obtain them in a pure state unless they are present to the extent of 5 % or more. We have thus been compelled to confine our attention to those constituents which are present in amounts too large for them to be present in the nucleus only, and therefore just as in the work on the proteins, we may regard the ether-soluble substances with which we are dealing as forming part of the cytoplasm.

The aim in this work has been to study as quantitatively as possible the ether-soluble extractives of the cytoplasm of one leaf only in order that the knowledge so obtained may be of possible use later in a similar study with small preparations from different species of leaves. With small quantities of material, little can be done save to determine the usual fat constants, and the results of the investigation into a larger quantity of similar fat will help to make the interpretation of those constants more of a probability and less of a gamble. The green leaves of unheaded cabbage—*Brassica oleracea*—were chosen for this work because they can be obtained throughout the year. Further, in this initial study, it has been desired to enquire in greater detail into the nature of the phosphorus-containing substances soluble in ether, and as preliminary investigation into the fats of a number of species of leaves seemed to show that the cabbage leaf was richer in ether-soluble phosphorus than the leaves of the other species which were used, that leaf was finally chosen.

#### *Details of preparation.*

After the separation of the leaves and the removal of their thick mid-ribs, the material was coarsely minced, mixed with an equal volume of distilled water, then finely minced and finally squeezed through fine silk. The residue of cellular material, after being treated with water, was again minced and squeezed out as before. Previous experimental work has shown that this deep green solution contains most of the vacuole constituents and part of the cytoplasm, the latter being dispersed in colloidal form, and that it does not contain any cell-wall material. The green juice is then heated to 70° when the cytoplasmic material is coagulated, and on cooling as rapidly as possible it settles as a green amorphous mass, leaving a clear brown fluid which is easily filtered through folded filters. The coagulated cytoplasm was enveloped in stout filter cloth, placed in a Buchner press and submitted to a pressure which was slowly increased to 400 kg. per sq. cm. and maintained until no more liquid was expressed. The product thus obtained was a dark green hard mass which could be readily powdered in a mortar in spite of the fact that analysis showed that it still contained about 40 % water. Such material is in a form which can be directly extracted with ether.

Prepared by this method the crude mass of cytoplasmic material will contain also those substances present in the vacuole fluid which are precipitable by heat. Previous experimental work [Chibnall, 1923] has shown that these are present in relatively small amounts, and consist of protein and inorganic material. Consequently, as the ether-soluble substances of the cytoplasm and not the proteins are now under investigation, these latter may be disregarded. Further, water-soluble substances from the vacuole will be present in the water still retained by the preparation, but, since the final solvent used for extraction of the fat is anhydrous ether, the presence of these substances will not affect the composition of the material obtained. A more

refined method of preparing cytoplasm has been already described [Chibnall and Grover, 1926], but on account of its more lengthy nature it could not be used in the present research. The essential point was to obtain the ether-soluble substances of the cytoplasm in large quantities, and the presence of vacuole protein and salts will not affect the question.

The material was extracted with ether in a Soxhlet apparatus with frequent changing of the receiving flasks, until the final extract was virtually colourless.

It was considered wise to make a study of the rate at which the fat was removed by ether, and the following table shows the weights of fat obtained during the various stages of a typical extraction.

Table I. *Showing the rate of extraction of the ether-soluble substances from 149 g. of cabbage leaf cytoplasm.*

Batch F, 9.5 kg. of leaves picked May 20, 1926.

Number of extract	Duration of extraction hours	Weight of extract g.
1	16	17.0
2	24	0.6
3	24	0.15
After regrinding of the powder:		
4	24	0.23
5	24	0.02

Schulze and Steiger [1889] reported that the phospholipins can only be completely removed from seed material if the ether extraction is followed by successive extractions with alcohol at 60°. It became of interest to see whether this finding applied also to the cytoplasmic phospholipins of the leaf. Accordingly after the exhaustive extraction with ether tabulated above, the residue was treated three times with large volumes of alcohol maintained at 60° and renewed every half hour. The combined alcohol extracts were evaporated to dryness at low pressure, and the residue treated with anhydrous ether. The total weight of this extract was 0.88 g. Finally, to ensure that there was no appreciable amount of fatty material left in the residual powder, the whole of the latter was hydrolysed for 3 hours with N HCl on a boiling water-bath. The mass was filtered and the residue washed several times with alcohol and then with ether. The alcohol-ether washings were combined with the ether extract of the filtrate and after thorough washing with water to remove alcohol, were evaporated to dryness. The weight of fatty acid so obtained was 0.69 g.

It will be seen that the extraction by ether is virtually completed at the end of 40 hours, when an extract weighing 17.6 g. has been obtained. Subsequent treatment of the residue with alcohol yielded 0.88 g. and hydrolysis 0.69 g. We therefore adopted ether extraction for 40 hours as our routine method, and the relatively small amount of material which can be obtained by subsequent treatment with alcohol and by hydrolysis has been ignored in this paper. It may be mentioned in passing that the alcohol extract does

not yield any quantity of phospholipin as Schulze showed to be the case in seed material.

The ether extracts contained a little water which had been removed by the ether from the coagulum. It has been our experience that the presence of water in such a solvent as ether will allow a small but definite amount of inorganic material and amino-compounds to go into solution. Since subsequent work on the extract was carried out with a view to determining the nature of the phosphorus-containing substances present, and since the percentage of nitrogen in those bodies is very low, it was considered essential to evaporate every crude extract to dryness *in vacuo* and make an anhydrous ether extract from it<sup>1</sup>. There always remained behind a small amount of material undissolved by that solvent, and this material was rejected.

*Preliminary analysis of the extracted cytoplasm.*

Tables II and III give details of the various samples of cytoplasm prepared. The method employed for the preparation of the cytoplasm in bulk is such that much of the cytoplasm is left behind in the cell residues. The amount actually obtained is dependent on the thoroughness with which the leaves were ground: only those cells which have been torn open will have their cytoplasm dispersed into colloidal solution. Some information may be obtained regarding the percentage of cytoplasm extracted by a determination of the protein content of the original leaf and of the cytoplasmic coagulum respectively. If such a rough method of calculation be adopted, it is only with the knowledge that it may involve an error of 5 %.

Table II. *Details of cytoplasm prepared from green leaves of cabbage.*

(Batch E, 20 kg. of leaves picked April, 1926.)		
Total leaf solids		2550 g.
" N		67 g.
" water-soluble N		23.9 g.
" protein-N		43.1 g.
" protein (N × 6.25)		269.3 g.
" cytoplasm (calculated)		405.1 g.
Percentage of total cytoplasm extracted		47 %
Extracted cytoplasm		
	g.	% total leaf solids
Protein (N × 6.25)	129	5.73
Ether-soluble substances	39	1.73
Ash	12	0.53
Undetermined	14	0.62
	194	8.61

From Table III it will be seen that the percentages of protein and ether-soluble substances in the various preparations are very constant for biological material. Further the protein : ether-soluble substances ratio is always about 3 : 1. These concordant figures are good evidence that the samples of material with which we are dealing are truly representative of the cytoplasm as a

<sup>1</sup> In this and subsequent papers the expression anhydrous ether means "0.720 ether" dehydrated by sodium.

whole. Thus, to consider extreme cases, in batches D and H, where the percentage of cytoplasm extracted was 41 and 28 respectively, the ratio is the same. Further evidence as to the uniformity of the different samples will be obtained by a study of the figures in Table IV which deal with the composition of the ether-soluble substances.

Table III. *Analyses of cytoplasm prepared from several different batches of green leaves of cabbage.*

	Autumn sown picked April and May				Spring sown picked August	
	D	E	F*	G	H	M
Weight of fresh leaves, kg.	20	20	9.5	9.0	32	50
	g.	g.	g.	g.	g.	g.
Weight of cytoplasm extracted	167.6	194.4	79.6	70.6	194.0	389.2
Protein (N $\times$ 6.25)	110.3	129.0	50.9	44.1	122.0	223.6
Ether-soluble substances	35.4	39.0	18.0	14.2	40.0	75.4
Ash	9.5	12.4	2.7	3.8	14.2	40.7
Undetermined	12.4	14.0	8.0	8.5	17.8	49.5
Ratio protein/ether-soluble substances	3.1	3.3	2.83	3.1	3.1	3.0
Composition of cytoplasm:	%	%	%	%	%	%
Protein (N $\times$ 6.25)	65.8	66.4	63.9	62.4	62.9	57.2
Ether-soluble substances	21.8	20.1	22.6	20.1	20.6	19.3
Ash	5.7	6.4	3.4	5.4	7.3	10.5
Undetermined	7.4	7.1	10.1	10.1	9.2	13.0
Percentage of total cytoplasm extracted	41	47	39	36	28	32

\* Exhaustive ether extraction.

A point worthy of notice is that the ether-soluble substances constitute one-fifth by weight of the total cytoplasmic solids. The ether extract on evaporation to dryness yielded a solid fat deeply pigmented with chlorophyll. We should have preferred to have removed the chlorophyll from the coagulum before the extraction of the fat. Since it was desired to obtain the fat in a state as little altered as possible from that in which it occurred in the leaf, the use of chemical methods of separating the chlorophyll was rendered impossible. Though many attempts were made to extract the pigment by the use of solvents, these proved fruitless, because all those solvents which will remove chlorophyll will dissolve some part of the fat. Therefore, in order to keep the work as quantitative as possible, the chlorophyll and fat were weighed together. The presence of this chlorophyll has necessarily modified the constants which would have been obtained had the fat been chlorophyll-free. Evidence will be adduced in a later paper, however, to show that the difficulties caused by the presence of the pigment can be overcome, and an approximate estimate of its amount and its effect on various determinations made.

The figures given in Table IV define the general characteristics of the ether-soluble substances. The percentage of material given under I was calculated by weighing the precipitate obtained when a solution of these substances in

dry ether was treated with 4 volumes of acetone. This high percentage figure for acetone-insoluble material does not represent phospholipins, as might be expected. It will be shown subsequently that not more than half of the weight of this fraction is due to substances containing phosphorus, and that these substances are nitrogen-free and therefore not phospholipins in the strict chemical sense. The other components of this fraction, which do not contain phosphorus, consist of one or more hydrocarbons and alcohols. The acetone treatment was found to remove all the phosphorus-containing substances and the figures given for the percentage of phosphorus-free material in II are obtained by difference.

The filtrate from I was evaporated to dryness *in vacuo*; on this material determinations of iodine value (Wijs) and saponification value were carried out. Attention is drawn to the very high iodine value and to the fact that the low saponification value is due not to the presence of fatty acids of very high molecular weight, but to the large percentage of unsaponifiable matter.

Table IV. *Showing the general characters of the ether-soluble substances of the cytoplasm.*

(All weights given are percentages of total ether extractives.)

	D	E	F	K
I. Weight of material precipitated by acetone	41.5	42.0	41.0	44.0
II. Weight of phosphorus-free fatty material	48.5	58.0	59.0	56.0
Iodine value (Wijs)	141	147	152	154
Saponification value	151	158	152	149
III. Weight of unsaponifiable matter	16.9	16.5	17.0	24.1
Iodine value (Wijs)	113	117	119	105
Weight of sterol	3.63	2.65	3.70	3.80
IV. Weight of fatty acids	23.2	23.1	23.0	15.0
Iodine value (Wijs)	173.5	203.5	201	206
Neutralisation value	209	206	198	197

The unsaponifiable matter was determined by the method already described [Drummond, Channon and Coward, 1925], the second saponification being carried out with sodium ethylate in alcohol, which is particularly necessary in dealing with plant materials containing definite quantities of the esters of the higher alcohols. The sterol content was determined by the digitonin method [Windaus, 1910].

The fatty acids were prepared after the removal of the unsaponifiable matter and were rendered free from chlorophyll degradation products.

The figures given in Table IV indicate how very constant the various values obtained from different batches of materials are. This confirms the results recorded in the previous tables and suggests that the fraction of cytoplasm obtainable is quite representative of the whole.

## SUMMARY.

The difficulties of preparing the ether-soluble substances of leaves are discussed. A method is given for preparing in bulk the ether-soluble substances of leaf cytoplasm, and the fat constants are given for several preparations made from cabbage leaves. The general uniformity of the results obtained is taken as evidence that the portion of cytoplasm extracted for analysis is representative of the whole.

## REFERENCES.

- Chibnall (1923). *J. Biol. Chem.* **55**, 333.  
Chibnall and Grover (1926). *Biochem. J.* **20**, 108.  
Drummond, Channon and Coward (1925). *Biochem. J.* **19**, 1047.  
Schutze and Steigor (1889). *Z. physiol. Chem.* **13**, 365.  
Windaus (1910). *Z. physiol. Chem.* **65**, 110.

# XXXIII. THE ETHER-SOLUBLE SUBSTANCES OF CABBAGE LEAF CYTOPLASM.

## II. CALCIUM SALTS OF GLYCERIDEPHOSPHORIC ACIDS.

By ALBERT CHARLES CHIBNALL  
AND HAROLD JOHN CHANNON (*Beit Memorial Research Fellow*).

*From the Departments of Physiology and Biochemistry,  
University College, London.*

(Received January 3rd, 1927.)

### INTRODUCTION.

In the previous paper the methods for preparing the ether-soluble substances of the cytoplasm from leaf cells on a large scale were described, and an account given of the general nature of the substances obtained from the green leaves of unheaded cabbage.

Determinations of the phosphorus content of the fatty material showed that this element is present in varying amounts. Thus batches *E*, *F*, *G* and *H* contained respectively 1.0, 1.6, 1.89, 1.1 % of phosphorus. It was mentioned in that communication that if four volumes of acetone be added to the solution of the ether-soluble substances in anhydrous ether in order to remove the phospholipin fraction, a large proportion of the material was precipitated. Thus from different preparations, 41, 42, 41 and 44 % of the ether-soluble material was precipitated in this way. Although the amount of phosphorus in the batches varied considerably, it will be seen that the amount of precipitate obtained by the addition of excess of acetone was constant. Now since the phosphorus was found to be almost quantitatively removed from the ether solution by precipitation with acetone, it follows that the phosphorus content of the acetone precipitate must vary in proportion to the phosphorus content of the ether solution from which it is derived. If we assume, therefore, that only one compound containing phosphorus is present in the ether solution, it follows that the acetone precipitate must necessarily contain at least one substance which is phosphorus-free. Actually it will be shown that there are present in it at least one solid hydrocarbon and one higher alcohol, both of which can be removed by treatment with boiling acetone or alcohol and subsequent filtration. It is thus apparent that the constant percentage of the ether-soluble substances precipitated from dry ether solution by the

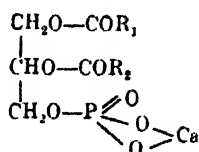


acetone is fortuitous, because this precipitate is made up of at least three substances which happen to be present in varying amounts, but the sum total of which is constant.

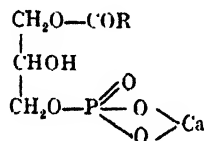
This communication deals only with the material which remains undissolved when the precipitate obtained by adding excess of acetone to the ether solution has been exhaustively extracted with boiling acetone. This fraction which is insoluble in hot acetone contains virtually all the phosphorus of the original ether extract; further every preparation made from different batches of leaves had a similar elementary composition. Thus the phosphorus content of the material from batches *D*, *F* and *H* was 4.9, 4.86 and 4.59 %; they contained 0.22, 0.23 and 0.22 % of nitrogen.

The appearance of this material, its rapid oxidation in air and the fact that it yielded about 70 % of its weight of fatty acids on hydrolysis, made it appear probable that it was of phospholipin character. Against this view however was the fact that its nitrogen content was very low, and it was certain that if this nitrogen were represented by lecithin, the bulk of the material must be of a different nature, as 0.2 % of nitrogen will only account for 10 % of the phosphorus present if calculated on the basis of lecithin. The possibility of the material being a uniform substance with an N:P ratio of 1:10 was considered highly improbable. It was therefore argued that this mixture probably contained two substances, one containing nitrogen and possibly phosphorus, but the bulk made up of a substance containing over 4 % of phosphorus and being nitrogen-free. If the molecule of the latter substance were built up in any way similarly to what we accept as the structure of lecithin, there must be some grouping to replace the choline radicle and the grouping must be nitrogen-free. The probability of it being a sugar was negatived, because hydrolysis with methyl alcoholic HCl gave no products which would reduce an alkaline copper solution; nor were any carbohydrate reactions obtained. It seemed probable, therefore, that a metal was replacing choline. The likely metals are K, Mg, Na, and Ca; and the last was the one actually found to be present in the molecule. Further, determinations of the calcium and phosphorus present, showed that in every preparation made, these substances bore an atomic ratio in all cases very close to unity.

Since the second substance has a phosphorus content of about 4 %, and a calcium content corresponding to the same number of atoms of that element as of phosphorus, and since the percentage of fatty acids obtained on hydrolysis was very similar to that which such a body as lecithin would yield, we based our work on the hypothesis that it was the calcium salt of a diglyceride-phosphoric acid of the type



The analyses quoted later show that the crude calcium salt is a mixture of at least two substances, one the calcium salt of the diglyceridephosphoric acid just outlined, the presence and structure of which has been definitely established, and the other probably the calcium salt of a monoglyceridephosphoric acid of the type



At least three-quarters of the material insoluble in boiling acetone consists of the calcium salt of the diglyceridephosphoric acid: it is for this reason that we have made the preparation and analysis of the free diglyceridephosphoric acid the main thesis of this paper. The possible presence of the monoglyceridephosphoric acid and the absence of lecithin are discussed in two appendices.

In discussing the analytical data which follow, it will be simpler if the figures required by the calcium salt of distearyl glycerophosphoric acid are taken as the theoretical basis. This is not strictly correct, for as appears later the fatty acids present in the molecule are mainly unsaturated; and hence the values required by theory will not be in strict accord with those obtained experimentally. Since a number of fatty acids seem to be present, and the crude calcium salt is thus really a mixture of substances containing different fatty acids, there is no alternative but to adopt some basis for theoretical calculation, and figures for the calcium salt of distearyl glycerophosphoric acid have therefore been used.

#### *Isolation of the crude calcium salt.*

*Batch H.* Total weight of ether-soluble substances 62.3 g. : P 1.10 %; Ca 1.43 %. Ratio Ca to P is approximately 1:1.

*Fraction precipitable by acetone.* The fat was dissolved in 400 cc. of anhydrous ether and 1600 cc. of pure acetone added; the solution was left for 1 hour on ice and filtered. The residue was ground with acetone and refiltered. The mother-liquor was reduced *in vacuo* to a volume of 200 cc. and four volumes of acetone were again added and, after standing on ice overnight, the solution was filtered. The combined precipitates, which weighed 28.1 g., were redissolved in ether and again precipitated with acetone. Experiments with earlier samples had shown that repetition of this procedure, although reducing the weight of material finally obtained, makes no appreciable difference to its phosphorus content. In other words, both the phosphorus-containing substance and the other substances present have similar solubilities in the ether-acetone mixture. Another method of purification was thus necessary, and

finally exhaustive extraction with boiling acetone was adopted. The substances extracted by this hot solvent crystallise as a pale green powder as soon as the temperature of the filtrate falls to 40°. The acetone extraction of this 28.1 g. was continued therefore until no crystalline material appeared on cooling the filtrate. (The filtrate will always go cloudy on cooling even though all the crystalline substances have been removed, for the phosphorus compound has a definite, though small, solubility in the hot solvent.)

By this procedure two fractions were obtained.

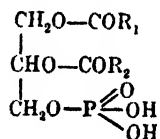
(1) The fraction insoluble in hot acetone. This fraction contains the calcium and the phosphorus. Weight 11.46 g. (18.6 % of total ether-soluble substance).

(2) The fraction soluble in boiling acetone. This consists chiefly of one or more hydrocarbons and alcohols, contaminated with traces of (1). The weight of this crystalline material was 13.0 g. (21 % of total ether-soluble substance); its nature will not be discussed further here, but will form the subject of a later communication.

*Crude calcium salt H.* The fraction insoluble in hot acetone, which we shall call the crude calcium salt, gave the following analysis:

0.0727 g. substance : 0.1524 g. CO <sub>2</sub> , 0.0572 g. H <sub>2</sub> O, 0.0142 g. ash.							
0.4316 g.       "       0.0712 g. Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub> .							
0.4316 g.       "       0.0862 g. CaSO <sub>4</sub> and 0.0065 g. Fe <sub>2</sub> O <sub>3</sub> .							
0.0888 g.       "       required (micro-Kjeldahl) 1.60 cc. 0.01 N acid.							
	C	H	Ca	P	Ash	Fe	N
Found	57.16	8.74	5.88	4.59	19.54	1.08	0.25
Theory	63.10	10.10	5.39	4.18	17.20	0.0	0.0

It will be seen that although the calcium and phosphorus are present in the ratio 1:1 the values obtained for these elements are too high for the calcium salt of a saturated diglyceridephosphoric acid (containing two C<sub>18</sub> acids), and, accepting the existence of the latter, it is to be surmised that a second substance containing calcium and phosphorus is also present. A substance of the type postulated above will readily lose its calcium when shaken in ether solution with aqueous mineral acid; there will then be produced an acidic substance of the type



which is a diglyceridephosphoric acid and which we may term the free acid.

With a view to obtaining some information regarding the possible second substance in the crude calcium salt a portion of about 5 g. of the latter was dissolved in ether, shaken with dilute HCl several times and the ether solution was well washed with water. By this procedure there were obtained 3.62 g. of free acid.

*Crude free acid H:*

0.3625 g. substance :	0.0505 g. $Mg_2P_2O_7$ .	
0.0727 g.        ,,	required (micro-Kjeldahl) 1.50 cc. 0.01 N acid.	
	P	N
Found	3.87	0.29
Theory	4.40	0.0

These figures show that a large proportion of the phosphorus has become water-soluble and been removed. The significance of this, together with the reasons for the phosphorus value being so low, will be discussed later. 0.727 g. of this free acid in ether solution was shaken out several times with lime water and the ether solution washed, reduced to a volume of 10 cc., and four volumes of acetone added to precipitate the regenerated calcium salt.

*Regenerated crude calcium salt H:*

0.1340 g. substance :	0.0184 g. $Mg_2P_2O_7$ .	
0.1340 g.        ,,	0.0265 g. $CaSO_4$ .	
	P	Ca
Found	3.83	5.82
Theory	4.18	5.39

It will be seen from these figures that the calcium : phosphorus ratio is now 1 : 1.18, in other words excess of calcium is present. This high value for calcium suggested that some other calcium-phosphorus-fatty acid compound in the original crude salt had broken down on being shaken out with acid. As a result the phosphorus has passed into the aqueous layer and been lost, whereas the fatty acid has remained in the ether layer along with the diglyceridephosphoric acid. On conversion of the latter into the calcium salt this contaminating fatty acid has been converted into the calcium soap. Hence the loss of phosphorus and increase in calcium could be explained.

*Preparation of the lead salt of a diglyceridephosphoric acid.*

As a means of preventing the possible contamination of the regenerated product, it was decided to make the lead salt after the crude free acid had been first obtained. 2.6 g. of the crude calcium salt were converted into the corresponding free acid, and the latter in ether solution was shaken several times with an aqueous solution of lead acetate containing a little acetic acid. If some substance had been previously hydrolysed to yield fatty acids these would not be capable of decomposing lead acetate. Hence they would remain as free acids along with the lead salt of the diglyceridephosphoric acid in the ether, and when the salt was precipitated by the addition of four volumes of alcohol, they would remain in the ether-alcohol mother-liquor (see Appendix 2). (It was found that the ether-alcohol mother-liquor retained the deep red colour which had been characteristic of the solution of the crude and regenerated calcium salts mentioned above.) The precipitated lead salt was greyish and dissolved in ether to give a pale yellow-brown solution. On precipitating a second time the ether-alcohol mother-liquor was water-clear.

The yield of the lead salt after two precipitations from the ether-alcohol was 1.707 g.

*Lead salt of diglyceridephosphoric acid H:*

0.0755 g. substance : 0.1414 g.  $\text{CO}_2$ , 0.0493 g.  $\text{H}_2\text{O}$ , 0.0240 g. ash.  
 0.1707 g.        "        0.0550 g.  $\text{PbSO}_4$ .  
 0.1707 g.        "        0.0207 g.  $\text{Mg}_2\text{P}_2\text{O}_7$ .

	C	H	Pb	P	Ash
Found	51.07	7.38	22.02	3.43	31.60
Theory	51.47	8.14	22.80	3.41	32.33 (calc. as $\text{Pb}_2\text{P}_2\text{O}_7$ )

This lead salt was contaminated with 0.65 % of Fe determined colorimetrically by comparison with standard  $\text{Fe}_2(\text{SO}_4)_3$  treated with  $\text{K}_4\text{Fe}(\text{CN})_6$ : it also contained 0.17 % of N determined by the micro-Kjeldahl method. The significance of the presence of these elements will be discussed later (see Appendix 1).

These analytical results were considered satisfactory for a compound of such a type as this. This preliminary work established a method of purifying the crude calcium salt and the next batch of material was accordingly treated in the same way.

ANALYSIS AND CONSTITUTION OF THE FREE DIGLYCERIDEPHOSPHORIC ACID.

*Preparation of the free diglyceridephosphoric acid.*

*Batch M.* From 64 g. of original ether-soluble material, 7.80 g. of crude calcium salt were obtained by the method already described. This sample had Ca 6.13 %, P 4.78 %; Ca : P = 1 : 1. The whole of this salt in ether solution was shaken out with dilute sulphuric acid to convert it into the corresponding acid, which in turn was shaken with lead acetate containing a little free acetic acid. The washed ether solution was concentrated to 250 cc. and two volumes of absolute alcohol added. The lead salt was filtered off, redissolved in 70 cc. of anhydrous ether and reprecipitated by the addition of three volumes of absolute alcohol. The yield of purified lead salt was 5.00 g.

*Lead salt of diglyceridephosphoric acid M:*

0.0769 g. substance : 0.1432 g.  $\text{CO}_2$ , 0.0526 g.  $\text{H}_2\text{O}$ , 0.0234 g. ash.

	C	H	Ash
Found	50.06	7.49	30.00
Theory	51.47	8.14	32.30

4.80 g. of this salt yielded 3.51 g. of free acid.

*Diglyceridephosphoric acid M:*

0.0832 g. substance : 0.1997 g.  $\text{CO}_2$ , 0.0742 g.  $\text{H}_2\text{O}$ .  
 0.2140 g.        "        0.0334 g.  $\text{Mg}_2\text{P}_2\text{O}_7$ .  
 0.0507 g.        "        required (micro-Kjeldahl) 0.85 cc. 0.01 N acid.  
 (Fe 0.59 %)

	C	H	P
Found	65.48	9.91	4.34
Theory	66.57	10.94	4.40

*Analysis of the diglyceridephosphoric acid.* 3.06 g. of the acid dissolved in 40 cc. of mixed alcohol and ether were emulsified with 20 cc. of water and slowly run drop by drop into 200 cc. of boiling water containing 6 g. of barium hydroxide. The boiling was continued for 2 hours and on cooling the barium soaps were removed by filtration. The barium soaps were ground twice with water and filtered. The combined filtrate and washings were treated with  $\text{CO}_2$  to remove excess barium, the barium carbonate filtered off and washed with water. The combined filtrate was boiled to precipitate any bicarbonate, cooled and filtered. The final filtrate was reduced to a volume of 25 cc. *in vacuo*, and  $2\frac{1}{2}$  volumes of absolute alcohol added. The precipitate, which appeared as a curd, was washed with alcohol and ether and dried *in vacuo*. The yield of crude barium glycerophosphate was 0.73 g. (55 % of theory). A control experiment in which 1 g. of barium glycerophosphate, prepared from commercial glycerophosphoric acid, was used, gave a similar percentage yield when submitted to exactly similar treatment.

*Analysis of the barium glycerophosphate.* 0.73 g. of the salt dissolved readily in about 10 cc. of water, giving a slightly yellow and faintly opalescent solution. It was filtered and two volumes of absolute alcohol were added to the filtrate. The precipitate was curdy as before and after washing was dried at  $107^\circ$ . Yield 0.63 g.

0.0860 g. substance : 0.0368 g.  $\text{CO}_2$ , 0.0201 g.  $\text{H}_2\text{O}$ , 0.0624 g. ash.

0.1582 g. substance on ignition gave 0.1122 g. ash, equivalent to 0.0155 g. P or 9.81 %; converted to the sulphate it gave 0.1154 g.  $\text{BaSO}_4$ , equivalent to 0.0679 g. Ba or 42.86 %. This amount of Ba is equivalent to 0.1110 g.  $\text{Ba}_2\text{P}_2\text{O}_7$ .

	C	H	Ba	P
Found	11.66	2.59	42.86	9.81
$\text{C}_3\text{H}_7\text{O}_6\text{PBa}\frac{1}{2}\text{H}_2\text{O}$	11.40	2.53	43.42	9.81

It was necessary to boil the aqueous solution twice with charcoal before it became clear enough for the optical rotation to be determined.

$$[\alpha]_D^{15} = + 1.0^\circ (l = 2, c = 3).$$

Fränkel and Dimitz [1909] isolated a dextrorotatory barium glycerophosphate from brain cephalin and Trier [1913] a similar product from seed lecithin. In each case the salt was purified by repeated solution in water and precipitation with alcohol. Levene and Rolf [1919, 1926] have since shown that in both these cases the dextrorotation was due to an impurity which cannot be removed by this treatment. They purified the barium salt by conversion into the lead salt, decomposed the lead salt by means of  $\text{H}_2\text{S}$  and reconverted the glycerophosphoric acid into the lead salt. This operation was repeated until a laevorotatory solution was obtained. From this solution a barium salt was precipitated by means of alcohol; this salt had the normal laevorotation.

Owing to the small amount of barium salt which we obtained it was not possible to purify it by this method, so that the question of its rotation must

be left open until such time as we are able to obtain sufficient quantities of material for this purpose. From Levene and Rolf's work it is to be inferred that the barium salt, after the lead treatment, will be laevorotatory.

The original mother-liquor from which the barium glycerophosphate had been precipitated by alcohol was evaporated to dryness *in vacuo*. The residue weighed 0.073 g., the major part of which was barium glycerophosphate. Exhaustive extraction with absolute alcohol dissolved 0.021 g. If the small amount of nitrogen present in the free acid was due to lecithin then we should expect to find evidence of choline in this alcoholic extract. Actually it was found to give no precipitate with either alcoholic  $\text{HgCl}_2$  or  $\text{H}_2\text{PtCl}_6$ . All the fractions of the barium glycerophosphate mother-liquor were then united and the total nitrogen determined by Kjeldahl. 0.3 cc. of 0.1 *N* acid were required, equal to 0.00042 g. of nitrogen and equivalent to 0.014 % of the total weight of free acid taken for hydrolysis. The significance of this result is discussed in Appendix 1.

*The fatty acids of the diglyceridephosphoric acid.* The barium soaps obtained during the baryta hydrolysis just described were decomposed with HCl under ether, the ether extract was thoroughly washed with water and evaporated to dryness. Yield 2.30 g. or 74.9 % of the diglyceridephosphoric acid taken (theory for  $\text{C}_{18}$  saturated acids 2.48 g. or 80.7 %).

0.1654 g. dissolved in abs. alcohol were neutralised by 0.81 cc. of 5/7 *N* HCl. Neutralisation value 196.

0.0920 g. absorbed 0.1254 g. iodine (Wijs). Iodine value 136.

These figures for the neutralisation and iodine values are in good agreement with those obtained in earlier (unrecorded) experiments. The amount of the fatty acids at our disposal was far too small for any reliable data to be obtained as to their nature. It seemed worth while however to attempt to determine whether they are in any way parallel to those of lecithin, which Levene has shown, in a large number of papers dealing with the acids of lecithins obtained from various sources, to consist of equal molecular proportions of unsaturated and saturated acids. Further, the work of that author and his colleagues on lyso-lecithins seems to exclude definitely the occurrence either of two saturated or two unsaturated acids in any one molecule of lecithin. The conclusion which Levene draws is that in each lecithin there is one saturated and one unsaturated acid.

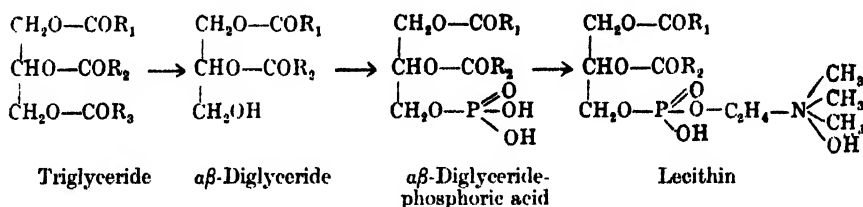
Accordingly 1.8 g. of the fatty acids was converted into the lead soaps, which were extracted with ether. There remained insoluble in ether only a very small amount of material, which yielded on decomposition with acid 0.1 g. of saturated acid, which was hard and white. It would appear therefore that the saturated and unsaturated acids are not present in equivalent proportions, but that the unsaturated acids are present in far greater amount. This result, which will necessarily need confirmation on larger quantities of material, tends to show that this compound differs from lecithin in that in any one molecule there may be two unsaturated acids present.





salt similar to that described in this paper. Also Stern and Thierfelder [1907] reported that a phosphatide fraction from egg-yolk contained 3.96 % P, 1.37 % N and 1.03 % Ca. They were convinced that the calcium was present in organic combination.

Although the natural occurrence of a diglyceridephosphoric acid had not been reported, it has been suggested by Trier [1912] that the successive stages in the synthesis of plant lecithins are as follows:



If the work reported in this paper be substantiated it may prove to be a link in the above scheme.

One further point needs discussion. It will be remembered that in the preparation of the original coagulum, the expressed juice is heated to 70° to coagulate the cytoplasm, and the possibility of this process having caused the partial hydrolysis, and the substitution of the choline present in lecithin by calcium, remains to be considered. The  $p_{\text{H}}$  of the expressed juice was about 6, and it seemed improbable that heating a suspension of lecithin to 70° for a few minutes in a solution of such low effective acidity would cause such a reaction to occur, and to proceed to completion.

To prove this point a batch of 7 kg. of leaves was minced in the usual way and the green solution containing the colloidal cytoplasm filtered instead of being warmed to 70° to coagulate the cytoplasm. Part of the protein and all the ether-soluble material was retained in the upper region of the filter pad. This green slime was scraped off and pressed in the Buchner press to a hard cake. This material, which had thus been prepared without the application of heat, was directly extracted with ether; the ether extract weighed 14.5 g. and yielded 2.6 g. of crude calcium salt.

0.0972 g. substance : 0.2090 g.  $\text{CO}_2$ , 0.0759 g.  $\text{H}_2\text{O}$ , 0.0182 g. ash.  
 0.2596 g. " 0.0503 g.  $\text{CaSO}_4$ , 0.0032 g.  $\text{Fe}_2\text{O}_3$ .  
 0.2596 g. " 0.0414 g.  $\text{Mg}_2\text{P}_2\text{O}_7$ .

	C	H	Ca	P	Ash	(Fe)
Found	58.63	8.67	5.70	4.65	18.73	0.86

This result would make it appear certain that the calcium salt of the diglyceridephosphoric acid pre-existed in the leaf cell, and that it was not a product of phospholipin decomposition.

*Properties of the diglyceridephosphoric acid, and the preparation and properties of some of its salts.*

*Free acid.* Prepared by evaporation of the ether solution at a temperature not exceeding 30°, it is a pale brown sticky mass, which does not readily

absorb moisture if exposed to the air, but rapidly darkens due to oxidation. Thus prepared it is readily soluble in the usual organic solvents, especially ether. An alcohol or acetone solution can be considerably diluted with water without the formation of an emulsion. Slow decomposition occurs if the free acid or any of its salts are dried *in vacuo* at 100°, giving rise to products that are no longer soluble in ether.

*Lead salt.* Two analyses of this salt have already been quoted. A third preparation made from Batch G by the methods described above gave the following analysis:

0.0946 g. substance : 0.1735 g. CO <sub>2</sub> , 0.0608 g. H <sub>2</sub> O, 0.0293 g. ash.					
0.1642 g. " 0.0524 g. PbSO <sub>4</sub> and 0.0204 g. Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub> .					
	C	H	Pb	P	Ash
Found (Prep. G)	50.01	7.14	21.80	3.46	30.86
" ( " H)	51.07	7.38	22.02	3.43	31.60
" ( " M)	50.06	7.49	—	—	30.00
Theory	51.47	8.14	22.80	3.41	32.38

When precipitated by absolute alcohol from a solution in anhydrous ether and dried *in vacuo* at 30° it is obtained as brown flakes resembling gelatin, which become sticky under pressure. It is readily soluble in ether, from which it is almost completely precipitated by two volumes of alcohol or less completely by four volumes of acetone. The dry product darkens rapidly when exposed to the air.

*Barium salt.* Prepared from an ether solution of lead salt H by converting into the free acid and shaking with barium acetate.

0.2456 g. substance : 0.0322 g. Mg <sub>2</sub> P <sub>2</sub> O <sub>7</sub> .		
0.1228 g. " 0.0352 g. BaSO <sub>4</sub> .		
	Ba	P
Found	16.80	3.65
Theory	16.37	3.69

*Calcium salt.* Prepared from an ether solution of lead salt G by converting into the free acid and shaking with calcium acetate.

0.0488 g. substance : 0.1154 g. CO <sub>2</sub> , 0.0397 g. H <sub>2</sub> O, 0.0089 g. ash.			
	C	H	Ash
Found	64.47	9.04	18.03
Theory	63.10	10.10	17.17

The barium and calcium salts have properties similar to those of the lead salt.

## APPENDIX 1.

### *On the possible presence of lecithin.*

It will have been noticed that in all the preparations there has been present a small amount of nitrogen (0.15 to 0.3 %). This nitrogen, which persists and is not readily removed by the many purification treatments, presumably cannot be present in the molecule itself, as this would entail a molecular weight

of 7 or 8 thousand. The presence of nitrogen in the phospholipin fraction is of course to be expected, and we thought naturally that this nitrogen represented lecithin. Throughout the work, however, we have failed to obtain any evidence of the presence of that substance. On no occasion has a precipitate been obtained when treating an alcoholic solution of the free acid with alcoholic  $\text{CdCl}_2$ . (This test cannot be carried out on the salts which are insoluble in alcohol.) Further this free acid, containing nitrogen sufficient to account for, say, 10 % of lecithin, is very soluble in cold acetone. Another impurity has also persisted. The small quantity of iron present in the crude calcium salt is not removed when the latter is converted into the free acid. Again, after the hydrolysis with baryta in the preparation of the barium glycerophosphate the barium soaps formed a precipitate as they are insoluble in water. The fatty acids which were prepared by acidification of these soaps under ether contained practically all the nitrogen and iron present in the original substance. Hence, as most of the nitrogen was not water-soluble after hydrolysis, it cannot be present as choline, and this is again evidence of the absence of lecithin. Another interesting point arises here, namely, that the iron and nitrogen have appeared together in the same fraction of the hydrolysis products and this may indicate the presence of ether-soluble iron- and nitrogen-containing substances which had been unaffected by the hydrolysis, and which, being water-insoluble, have precipitated along with the barium soaps. The evidence thus seems to negative the possibility of lecithin being present in this ether extract of leaf cytoplasm—a very interesting result.

## APPENDIX 2.

### *The possible presence of the calcium salt of a monoglyceridephosphoric acid.*

The point which will now be discussed is the significance of the fact that when the crude calcium salt in ether solution is shaken with acid, part of the phosphorus originally present passes into the aqueous solution. This fraction was usually 20-40 %. As to the nature of the substance from which it is derived, we have no positive evidence, but a number of isolated observations seem to throw some light on the question. Firstly, if the regenerated calcium salt is again shaken out with acid there is no further loss of phosphorus, which would suggest that the phosphorus which passes into the aqueous solution is derived from a second phosphorus-containing body and not from the calcium salt of the diglyceridephosphoric acid. Secondly, the regenerated calcium salt obtained by decomposing the crude salt with acid, and shaking the free acid so obtained with lime water always gives a high value for calcium and a low value for phosphorus. We may surmise from this that whatever has been hydrolysed by the shaking with acid and thereby has lost its phosphoric acid, must have given rise at the same time to other products of a fatty nature, which are able to unite with the lime. We suspected that these acidic sub-

stances might be fatty acids, and this was confirmed to some degree as follows. When the free acid in ether solution is shaken with aqueous lead acetate the lead salt of the diglyceridephosphoric acid is formed, and can be precipitated by the addition of excess of alcohol to the ether solution. If it be filtered off, the mother-liquors on evaporation leave a residue consisting of small amounts of lead salt together with another substance. This latter can be removed by solution in cold alcohol, and can be titrated in that solvent with NaOH. From the neutralised product there was obtained from batch M, discussed above, 1.22 g. of crude fatty acid having a neutralisation value of 177. Hence the second substance, which breaks down on treatment with acid, contains calcium and phosphorus, and gives rise to fatty acids. Further, since the calcium and phosphorus in the original crude calcium salt are both above the theoretical for the calcium salt of a diglyceridephosphoric acid the other substance present must contain a high percentage of calcium and phosphorus. Since this latter gives rise to fatty acids on hydrolysis, it seems possible that the second substance may be the calcium salt of a monoglyceridephosphoric acid, which would contain Ca 8.42 %. P 6.53 %. The presence of this substance in the crude Ca salt would raise both the calcium and phosphorus, and it seems reasonable to suggest that if such a substance in ether solution were treated with aqueous acid, it would tend to undergo hydrolysis much more easily than would the compound containing two fatty acid radicles in the molecule. No figures have been quoted for the amount of phosphorus passing into the aqueous solution, for, when the calcium salt has been shaken with mineral acid and the ether solution of the free acid is being shaken with water to remove the last traces of mineral acid, emulsions always occur. These emulsions are difficult to break, and it has been our practice to do this by adding small quantities of alcohol. There is little doubt that some of the loss of phosphorus is due to the removal of small quantities of free acid owing to its solubility in water containing alcohol. This fact has made it seem useless to quote figures for the phosphorus of the aqueous extract.

We hope to investigate this calcium salt of a monoglyceridephosphoric acid in greater detail at a later date. It is possible that this supposed salt did not pre-exist in the leaf cell, but was formed from the calcium salt of the diglyceridephosphoric acid by the action of enzymes or heat.

#### SUMMARY.

The so-called phospholipin fraction—obtained by adding acetone to an ether solution of a fat—contains no phospholipins in the case of the ether-soluble substances of the cytoplasm of the cabbage leaf. All the phosphorus is present in combination with calcium, glycerol and fatty acids. Nitrogen is virtually absent.

The main constituent of the fraction is the calcium salt of a diglyceridephosphoric acid. The preparation and analysis of this acid, and the preparation and properties of some of its salts are given.

## REFERENCES.

- Fränkel and Dimitz (1909). *Biochem. Z.* **21**, 337.  
Hundeshagen (1883). *J. prakt. Chem.* (2) **28**, 219.  
Levene and Rolf (1919). *J. Biol. Chem.* **40**, 1.  
—— — (1926). *J. Biol. Chem.* **68**, 285.  
Stern and Thierfelder (1907). *Z. physiol. Chem.* **53**, 360.  
Trier (1912). Über einfache Pflanzenbasen und ihre Beziehungen zum Aufbau der Eiweissstoffe und Lecithine (Berlin).  
—— (1913). *Z. physiol. Chem.* **86**.  
Winterstein and Stegmann (1909). *Z. physiol. Chem.* **58**, 527.

## XXXIV. ANALYSIS OF PROTEINS. VIII. ESTIMATION OF CYSTINE IN THE MODIFIED VAN SLYKE METHOD.

By ROBERT HENRY ADERS PLIMMER AND JOHN LOWNDES.

*From the Chemical Department, St Thomas's Hospital Medical School, London.*

*(Received January 10th, 1927.)*

IN the shortened form of the Van Slyke method of analysis of proteins adopted by Plimmer and Rosedale [1925], in which the determination of the various amino-acids was carried out without removal of phosphotungstic acid from the solutions, the estimation of cystine was not attempted. The direct determination of cystine has now been investigated with satisfactory results.

The estimation of cystine in proteins by this method depends also upon its behaviour in the various stages of the process. Van Slyke [1911] found that only 50 % of the cystine, after hydrolysis with 20 % hydrochloric acid for 24 hours, was precipitated by phosphotungstic acid. It was shown by Hoffman and Gortner [1922] that cystine on boiling with hydrochloric acid was slowly changed into an "isomeric" form which was more soluble, was optically inactive, did not crystallise in the characteristic form of ordinary cystine, and formed a much more soluble phosphotungstate. These observations have been confirmed and extended. The sulphur content of the diamino-fraction really represents from 40 to 50 % of the cystine in the protein. Either a correction could be applied assuming that half the cystine was precipitated by phosphotungstic acid, or a determination of the sulphur content of the monoamino-fraction could be made for the amount of the non-precipitated "isomeric" cystine. This latter determination involves the assumption that no other sulphur-containing compound is present. In view of the isolation of another sulphur-containing amino-acid by Mueller [1923] the sulphur content of the monoamino-fraction cannot be regarded as being due only to "isomeric" cystine. It has been possible for us to estimate the sulphur content of the solutions of the several egg proteins analysed by Plimmer and Rosedale [1925]. The amounts of sulphur in the monoamino-fractions seem to bear no relation to the amounts in the diamino-fractions, but tend to indicate the presence of another sulphur compound which is not precipitated by phosphotungstic acid.

Though the sulphur estimation of the diamino-fraction does not represent the actual amount of cystine in a protein, its estimation is necessary in order to arrive at the amount of lysine. The presence of cystine in this fraction gives

erroneous values for arginine, as it gives off, according to Van Slyke [1911], 18 % of its nitrogen on boiling with 40 % caustic potash. The decomposition has been found to be rather less on boiling with 20 % caustic soda, under the conditions laid down for arginine by Plimmer [1916]. It is greater in the presence of phosphotungstic acid.

Plimmer and Rosedale [1925] found that the monoamino-fraction gave off ammonia on boiling with caustic soda and attributed its origin to arginine which had not been precipitated by phosphotungstic acid. The amount of sulphur in these fractions is too small to account for the quantity of ammonia. On standing for over 15 months these solutions have deposited small quantities of crystals. Their examination has shown them to consist of a mixture of diamino-acids and the solution has still given off ammonia on boiling with caustic soda. The monoamino-acids, glycine, alanine, tyrosine and proline have been boiled with caustic soda and do not evolve any ammonia. It has not yet been possible to test a hydroxyamino-acid, so that the origin of the ammonia still appears to be from non-precipitated arginine. It is most likely that part of the arginine of the protein is racemised on hydrolysis and that racemic arginine forms a more soluble phosphotungstate than the active arginine.

#### EXPERIMENTAL.

The cystine for these experiments was prepared from feathers. The feathers were hydrolysed by boiling with twice their weight of conc. hydrochloric acid for 6 hours. The acid solution was evaporated *in vacuo* to remove hydrochloric acid as far as possible. The residue was dissolved in water, filtered from black material, boiled with charcoal, again filtered, nearly neutralised with sodium hydroxide (Hoffman and Gortner) and treated with sodium acetate until neutral to Congo red (Folin). The brown product was purified by boiling in 5 % (vol.) hydrochloric acid with charcoal, filtering and precipitating with sodium acetate. The colourless product was then treated with alcohol saturated with hydrogen chloride to remove tyrosine [Plimmer, 1913] and crystallised several times from dilute hydrochloric acid by addition of sodium acetate. After drying in a desiccator, the cystine gave the following figures on analysis:

Found (mean of 5 analyses): N = 11.5 %, S = 26.3 %.

Calculated for  $C_6H_{12}O_4N_2S_2$ : N = 11.67 %, S = 26.67 %.

Both analytical figures thus indicated a purity of 98.6 %. The sample was found to leave no ash on incineration, and microscopical examination showed only the typical hexagonal crystals of cystine. It is not possible at present to explain the low analytical data for the purified cystine. It may be noted that Hoffman and Gortner obtained similar analytical figures for pure cystine indicating a purity of 97.5 %<sup>1</sup>.

<sup>1</sup> [Note added February 7, 1927.] Dr W. H. Hurttley has suggested to us that cystine pre-

I. *Estimation of cystine sulphur in presence of phosphotungstic acid.*

In the Van Slyke method of protein analysis 15 g. of phosphotungstic acid are used in precipitating the diamino-fraction. Known weights of cystine have been oxidised in presence of this large quantity of phosphotungstic acid by the Benedict-Denis method.

On treating the oxidised residue with dilute hydrochloric acid, it was found that the tungstic acid, produced during the incineration, gave a very fine white precipitate which settled out only slowly and was very difficult to separate by filtration. A further quantity of tungstic acid also came down on adding barium chloride for precipitation of the barium sulphate. This treatment was thus not a practical one for the purpose.

As tungstic acid forms a very insoluble yellow oxide on precipitation from strongly acid solutions, whereas it gives the white hydrated oxide in dilute solution, the next experiments were made using conc. hydrochloric acid for solution of the oxidised residue. The greater part of the tungstic acid was then obtained as yellow oxide which could be easily filtered off on a hardened paper and washed with conc. hydrochloric acid. On diluting the solution with water, a small quantity of the white oxide was precipitated; on warming it settled out and could be filtered off and washed with dilute hydrochloric acid. A clear solution was thus obtained for precipitation with barium chloride. The last traces of tungstic acid are not however always removed by this treatment and come down with the barium sulphate. These small amounts are easily soluble in dilute alkali and can be removed by washing the precipitate of barium sulphate with dilute ammonia.

As a routine, the procedure may be described as follows. The solution of cystine phosphotungstate (20 cc. diamino-fraction, or 50 cc. monoamino-fraction) is evaporated to dryness with 5 to 10 cc. of the Benedict-Denis reagent, preferably on a hot plate, until distinct charring is seen. The residue is then heated over a flame for 15 minutes. The oxidised material is boiled with 25 cc. of conc. HCl. After standing (most conveniently overnight) the solution is filtered from insoluble yellow tungstic oxide through a 9 cm.

evaporated from acid solution with sodium acetate may retain traces of acetic acid and thus give low analytical figures for nitrogen and sulphur.

A sample of cystine prepared by Dr Hurtleby by precipitation with sodium hydroxide and kindly supplied to us gave the following data:

0.1 g. gave 11.55}	11.65 cc. N/14 NH <sub>3</sub>	N = 11.65 %
0.1 g. gave 11.75}		
0.1 g. gave 0.1941 g. BaSO <sub>4</sub>		S = 26.65 %

On recrystallising our own specimen by precipitation from acid with sodium hydroxide, it gave the correct figures:

0.1 g. gave 11.65}	11.65 cc. N/14 NH <sub>3</sub>	N = 11.65 %
0.1 g. gave 11.65}		
0.1 g. gave 0.1930}	0.1936 g. BaSO <sub>4</sub>	S = 26.00 %
0.1 g. gave 0.1943}		

It thus appears that the low values for cystine nitrogen and sulphur are due to precipitation with sodium acetate and retention of acetic acid.



hardened paper and the precipitate washed with from 25 to 50 cc. of hot conc. HCl and then with hot 50 % HCl. The solution is diluted with 200 to 500 cc. of water. The white precipitate which forms settles out as yellow oxide on warming and is filtered off and washed with dilute HCl (1:1). The clear solution is precipitated with barium chloride. The barium sulphate is washed thoroughly with water and then with dilute ammonia to remove the last traces of tungstic acid. The final treatment of the barium sulphate is carried out by the Folin method of washing with alcohol, burning the paper in a crucible and heating to redness. The following results have been obtained:

0.25 g. cystine + 15 g. phosphotungstic acid gave 0.4806 g.  $\text{BaSO}_4$  } S = 26.36 %.  
 0.25 g. " " " " " 0.4794 g. " " }

These figures correspond with those for the cystine used above, S = 26.31 %.

## II. Estimation of cystine sulphur in cystine phosphotungstate.

As the main object of these experiments was the estimation of cystine in the phosphotungstic acid precipitate obtained in the modified Van Slyke procedure, known quantities of cystine (0.25 g.) in hydrochloric acid solution were precipitated by an excess of phosphotungstic acid in a total volume of 200 cc. Under these conditions the precipitate came down slowly. After standing for 5 days, the precipitate was filtered off on a special glass filter and washed with 5 portions of 10 cc. dilute hydrochloric acid. The precipitate was dissolved in sufficient caustic soda solution and the volume made up to 100 cc. 20 cc. portions of the solution were taken for total nitrogen and for sulphur determinations.

Mean			Mean		
N	{ 5.5    5.65 } { 5.65    5.6 }	5.6 cc. N/14 $\text{NH}_3$ .	S	{ 0.0930    0.0934 } { 0.0928    0.0928 }	0.0930 g. $\text{BaSO}_4$ .

For the corresponding quantity of 0.05 g. cystine the quantities should be 5.75 cc. N/14  $\text{NH}_3$  and 0.0958 g.  $\text{BaSO}_4$ .

Both the nitrogen and the sulphur estimations showed that 97.4 and 97.1 % of the cystine were precipitated. The figures correspond with those of Hoffman and Gortner, who determined the extent of the precipitation by nitrogen determinations.

The filtrate from the precipitate of the cystine phosphotungstate was also examined for nitrogen and sulphur: 50 cc. of the filtrate made up to 250 cc. gave:

0.1 and 0.2 } cc. N/14  $\text{NH}_3$       0.0022 and 0.0008 g.  $\text{BaSO}_4$ .  
 0.15 " 0.1 }

The nitrogen was thus equal to 2.5 % of the total nitrogen. The amounts of barium sulphate were insufficient for accurate weighing.

## III. Behaviour of cystine during hydrolysis.

The observations of Van Slyke [1911] and of Hoffman and Gortner [1922] upon the action of hydrochloric acid upon cystine have been repeated and extended. One portion of 1 g. cystine and one portion of 2.5 g. cystine were boiled for 36 hours under a reflux with 100 and 250 cc. of 25 % HCl. The

solutions became brown in colour, deposited a dirty brown precipitate and gave a distinct odour of hydrogen sulphide. The excess of hydrochloric acid was distilled off *in vacuo* and the volumes made up to 100 cc. and 250 cc. A complete series of analyses was then carried out by the Plimmer-Rosedale procedure, using quantities of the solutions containing 0.25 g. cystine. The following are the data:

	I		II	In % of	
	Total N			Total N	Total S
Hydrolysed solution	5.6		5.6 cc. N/14	97.4	98.0
	5.7		5.6 cc. "		
	0.4700		0.4711 g. BaSO <sub>4</sub>		
	0.4692		0.4622 g. "		
Amide N	1.3	1.2	1.25 cc. N/14	4.3	—
Humin N	0.9	0.65	0.85 cc. "	2.7	—
Phosphotungstic precipitate:					
20 cc. out of 100 cc.	2.1	2.2	2.0 cc. "	—	—
	0.040	0.0412	0.0386 g. BaSO <sub>4</sub>	36.5	41.8
Phosphotungstic filtrate:					
50 cc. out of 250 cc.	3.2	3.25	2.85 cc. N/14	—	—
	0.0538	0.0542	0.0528 g. BaSO <sub>4</sub>	53.9	56.2

Cystine is thus decomposed by boiling with acid. About 7 % of its nitrogen was lost as amide and humin nitrogen. The sulphur content of the diamino-fraction represents about 40 % of the cystine sulphur; 56 % of the sulphur is found in the monoamino-fraction.

#### IV. *Estimations of the sulphur content of egg proteins.*

The remainders of the diamino- and monoamino-fractions of the egg proteins analysed by Plimmer and Rosedale [1925] have been analysed for their sulphur content by the method described above. The figures for barium sulphate have been changed to figures for cystine nitrogen by using the equivalent of 1 mg. BaSO<sub>4</sub> = 0.06 mg. cystine N. The data are:

	Diamino-fraction 100 cc. g.	Monoamino-fraction 250 cc. g.
Egg-yolk	0.0004	0.0170
Egg-white	0.0038	0.0124
Ovomucoid	0.0026	0.0183
Egg-membrane	0.0065	0.0133
Casein	0.0000	0.0010
Gelatin	0.0002	0.0075

It is of interest to notice the low amount of cystine nitrogen in the diamino-fraction of egg-yolk. The two phosphoproteins, caseinogen and vitellin, thus resemble each other also in their cystine sulphur content.

From the results of the analyses on boiling cystine with hydrochloric acid it might have been expected that the sulphur content of the monoamino-fraction would have been  $\frac{3}{2}$  times that of the diamino-fraction, but except in the case of the egg-membrane the amount is considerably greater. It may therefore be inferred that another sulphur compound is present in all the proteins. Egg-membrane appears to contain mostly cystine sulphur.

V. *The presence of arginine in the monoamino-fraction of the egg proteins.*

The monoamino-fractions of the egg proteins and caseinogen analysed by Plimmer and Rosedale [1925] have, on standing for over 15 months, deposited small quantities of crystals. These crystals have been filtered off, dissolved in dilute soda, and analysed for total nitrogen and arginine nitrogen and in one case cystine sulphur. The filtrates have also been analysed for total and arginine nitrogen. The figures are:

	Volume of remaining solution cc.	Original		Filtrate		Precipitate		
		T.N.	Arg. N.	T.N.	Arg. N.	T.N.	Arg. N.	Cyst.
Caseinogen	1875	0.2086	0.0052	0.2114	0.0056	0.0287	0.0098	—
Egg-yolk	600	0.1666	0.0126	0.0166	0.0098	0.0042	0.0036	—
Egg-white	610	0.2016	0.0126	0.1974	0.0098	0.0042	0.0036	—
Ovomucoid	740	0.1946	0.0238	0.1862	0.0238	0.0140	0.0028	—
Egg-membrane	710	0.1960	0.0168	0.1890	0.0154	0.0182	0.0049	0.0020

There has been a small diminution, as expected, in the total nitrogen of the solutions and little change in the amount of arginine nitrogen. The precipitate consists only in part of arginine phosphotungstate. The amounts of "isomeric" cystine in these solutions (section IV) are not sufficient to account for the whole of this arginine nitrogen.

The possibility that the ammonia arose from monoamino-acids, though they were shown to be stable to caustic potash by Van Slyke, has again been tested by boiling solutions of glycine, alanine, proline, and tyrosine with 20 % caustic soda. No ammonia was evolved in any of these experiments. Specimens of hydroxyamino-acids have not been available for testing with caustic soda. The original conclusion that the ammonia arose from non-precipitated arginine is thus strengthened. It is possible that the arginine in the solution comes from racemic arginine which may form a more soluble phosphotungstate.

VI. *Action of sodium hydroxide on cystine.*

The action of caustic potash upon cystine was tested by Van Slyke [1911], who found that 18 % of its nitrogen was given off. To complete the series of observations upon the behaviour of cystine in the Van Slyke method, the action of 20 % caustic soda for 6 hours, the conditions found by Plimmer [1916] for the decomposition of arginine, has been tried upon cystine, "hydrolysed" cystine, cystine precipitated by phosphotungstic acid and unprecipitated "isomeric" cystine. The results were as follows:

Solution boiled with 20 % caustic soda	Total N of solution cc. N/14	Ammonia evolved cc. N/14		Mean	% of total N
10 cc. 0.1 % pure cystine	11.5	1.0	1.2	1.1	9.6
10 cc. 0.1 % hydrolysed cystine	11.2	1.5	1.3	1.3	11.8
20 cc. cystine phosphotungstate in soda	2.1	0.5	0.4	0.45	21.4
50 cc. filtrate from phosphotungstate	3.1	0.4	0.45	0.4	13.7

The decomposition of pure cystine is thus less with 20 % soda than found by Van Slyke with 40 % potash. The amount is a little greater after hydrolysis. In presence of phosphotungstic acid, the amount of decomposition is greater. This decomposition of cystine by boiling with soda will give results for arginine which are too high. A deduction of one-fifth of the cystine nitrogen should be made before calculating the arginine nitrogen.

#### SUMMARY.

1. The sulphur of cystine can be estimated in the presence of phosphotungstic acid by a suitable alteration of the Benedict method of determining sulphur.

2. 97 % of pure cystine is precipitated by phosphotungstic acid.

3. Cystine is changed by boiling with acids, losing 7 % of its nitrogen; only 40 % is then precipitated by phosphotungstic acid.

4. Cystine, on boiling with caustic soda, loses 10 % of its nitrogen as ammonia. The decomposition reaches 20 % after boiling with acid and in presence of phosphotungstic acid.

5. The amounts of cystine sulphur in the egg proteins and caseinogen have been determined. The figures indicate the presence of another sulphur compound in these proteins.

6. The probable presence of arginine in the monoamino-fractions obtained in the Van Slyke method of analysis is emphasised by its precipitation as phosphotungstate when the solutions are kept. It is possibly racemic arginine.

We wish to acknowledge a grant to one of us from the Government Grant Committee of the Royal Society to cover the expenses of this work.

#### REFERENCES.

- Hoffman and Gortner (1922). *J. Amer. Chem. Soc.* **44**, 341.  
Mueller (1923). *J. Biol. Chem.* **56**, 157.  
Plimmer (1913). *Biochem. J.* **7**, 311.  
— (1916). *Biochem. J.* **10**, 115.  
Plimmer and Rosedale (1925). *Biochem. J.* **19**, 1015.  
Van Slyke (1911). *J. Biol. Chem.* **10**, 15.

# XXXV. CHANGES IN THE AMINO-ACIDS IN THE PROTEINS OF THE HEN'S EGG DURING DEVELOPMENT.

By ROBERT HENRY ADERS PLIMMER AND JOHN LOWNDES.

*From the Chemical Department, St Thomas's Hospital Medical School, London.*

*(Received January 10th, 1927.)*

OF the various changes which take place in the hen's egg during development into the chick those occurring in the protein have not yet been thoroughly investigated. The excellent review of all the work on "The Metabolism of the Developing Egg" by Needham [1925] does not refer to any investigation of the proteins. Several papers have been published upon changes in the ammonia and nitrogen content, and Needham [1926, 1] has published data upon the urea content of the developing egg, and he has also studied the uric acid and non-protein nitrogen content [1926, 2]. Fiske and Boyden [1926] have also worked upon the nitrogen metabolism of the chick embryo.

Sendju [1925] has determined the tyrosine and tryptophan content of the developing egg by colorimetric methods and the diamino-acid content by Kossel's method. A decrease was found in the tyrosine and tryptophan content, but no definite changes in the diamino-acid content.

With the improvements made in the Van Slyke method by Plimmer and Rosedale [1925] it was hoped it would be possible to detect changes in the amino-acid content which would indicate the origin of the ammonia, urea, uric acid and other simpler compounds.

## EXPERIMENTAL.

Previous experiments upon the changes in the constituents of eggs during development had shown that no marked alteration was noticeable until about the 11th day of incubation. No attempt was therefore made to analyse the amino-acids at daily intervals. The fresh egg, the egg at the stage of 15 days' incubation and the hatched chick were used so as to secure information at the beginning, at the middle and at the end of the metamorphosis. The eggs in these experiments were from Light Sussex hens fed upon a diet of vitamealo, middlings and maize. Three eggs were used at each stage of the development. Sufficient material was thus obtained to allow of several analyses of the amino-acids by the Van Slyke method.

The contents of the fresh eggs, the eggs at 15 days, and the chicks after chloroforming and cutting up into small pieces were placed in 400 to 500 cc. of alcohol and treated in a similar way to that described by Plimmer and Scott [1909] in order to separate fats and lecithin and to coagulate the protein. The material after standing some days in alcohol was warmed and extracted first with alcohol and then with ether in a muslin bag suspended in a special extractor. The amounts of coagulated protein so obtained were:

From fresh eggs	17.0 g.
„ eggs at 15 days' incubation	20.6
„ hatched chicks	19.3

The alcohol and ether extracts were made up to 1000 cc. and the nitrogen in them determined by Kjeldahl's method. The amounts were:

From fresh eggs	0.1288 g.
„ eggs at 15 days' incubation	0.1344
„ hatched chicks	0.1190

These figures, if calculated per 100 g. of protein, show a decrease in the amount of alcohol- and ether-soluble nitrogen, but they probably have no relation to any changes in the proteins.

The coagulated protein at each stage was hydrolysed with 200 cc. of 25 % hydrochloric acid for 36 hours. The solution was evaporated *in vacuo* to remove hydrochloric acid as far as possible and the residue dissolved in water and made up to 520 cc.

The amino-acids were determined in 100 cc. portions of these solutions by the Van Slyke method as modified by Plimmer and Rosedale. Four analyses were made at each stage. The residual diamino- and monoamino-acid solutions were then combined and the analyses repeated. Sulphur estimations were also made by direct determination, as shown possible by Plimmer and Lowndes [1927].

The series of analyses has been repeated with one egg at each stage. These eggs were also from Light Sussex hens, but fed upon white rice, dried yeast, fish meal and cod-liver oil, from group XLV of birds in other experiments on nutrition.

The amounts of protein obtained were:

From a fresh egg	5 g.
„ an egg at 15 days' incubation	5
„ a hatched chick	7

After hydrolysis the solutions were made up to 250 cc. and two analyses of the amino-acids were made. The residues were combined for another estimation and a sulphur estimation.

Although it had been found by Plimmer and Phillips [1924] that the tyrosine content could not be determined by bromination on account of the presence of cystine, or some other compound which reacts with bromine, it was of some interest to ascertain the absorption of bromine by the mono-amino-fraction at each stage of development. This estimation has been made for us by Mr L. R. Bishop.

The following are the data, all calculated in terms of nitrogen per 100 cc.:

Exp. 1.	Total N. Stage: 0								0.4578 g.			
	" 15 days								0.4928			
	" hatched chick								0.4788			
	Diamino N								Monoamino-N			
	Anide	Humm	Total	Amino	Non-amino	Arginine	Histidine	Ly-sine	Total	Amino	Non-amino	Arginine
Stage: 0												
(1)	0.0372	0.0073	0.1190	0.0708	0.0482	0.0651	0.0009	0.0548	0.2954	0.3150	—	0.0224
(2)	0.0378	0.0067	0.1162	0.0690	0.0472	0.0644	0.0016	0.0534	0.2926	0.2995	—	0.0210
(3)	0.0377	0.0067	0.1176	0.0642	0.0534	0.0616	0.0108	0.0452	0.2898	0.3050	—	0.0196
(4)	—	0.0063	0.1176	0.0683	0.0483	—	—	—	0.2954	0.3050	—	0.0210
Mean	0.0376	0.0067	0.1176	0.0683	0.0483	0.0637	0.0023	0.0516	0.2933	0.3061	—	0.0210
Residual solution	—	—	0.1190	0.0672	0.0518	0.0678	0.0014	0.0498	0.2926	0.3102*	—	0.0196
Stage: 15 days												
(1)	0.0435	0.0092	0.1302	0.0733	0.0569	0.0721	0.0042	0.0539	0.3010	0.3115	—	0.0210
(2)	0.0454	0.0087	0.1330	0.0737	0.0593	0.0707	0.0094	0.0529	0.2996	0.3150	—	0.0196
(3)	0.0423	0.0092	0.1316	0.0789	0.0527	0.0742	0.0044	0.0618	0.3021	0.3242	—	0.0168
(4)	0.0399	0.0090	0.1302	0.0796	0.0506	0.0707	0.0036	0.0631	0.2996	0.3160	—	0.0151
Mean	0.0428	0.0090	0.1312	0.0764	0.0548	0.0719	0.0013	0.0580	0.3006	0.3167	—	0.0182
Residual solution	—	—	0.1358	0.0721	0.0637	0.0756	0.0105	0.0497	0.3024	0.3212*	—	0.0168
Stage: hatched chick												
(1)	—	0.0077	—	—	—	—	—	—	0.2912	0.2780	0.0132	0.0154
(2)	0.0442	0.0080	0.1302	0.0679	0.0623	0.0742	0.0100	0.0460	0.2881	0.2833	0.0051	0.0151
(3)	0.0364	0.0078	0.1260	0.0753	0.0507	0.0833	0.0177	0.0604	0.2884	0.2845	0.0039	0.0154
(4)	0.0381	0.0085	0.1316	0.0676	0.0640	0.0805	0.0004	0.0507	0.2912	0.3002	—	0.0168
Mean	0.0396	0.0080	0.1293	0.0703	0.0590	0.0793	0.0006	0.0506	0.2898	0.2865	0.0033	0.0158
Residual solution	—	—	0.1330	0.0679	0.0651	0.0819	0.0055	0.0456	0.2870	0.2790*	0.0060	0.0151

\* On repeating at a later date: stage 0, 0.2905; 15 days, 0.2955; hatched chick, 0.2716.

Exp. 2.	Total N. Stage: 0								0.2912 g.			
	" 15 days								0.2646			
	" hatched chick								0.2464			
	Stage: 0											
(1)	0.0280	0.0053	0.0700	0.0435	0.0265	0.0309	0.0051	0.0352	0.1834	0.1730	0.0104	0.0112
(2)	0.0290	0.0055	0.0700	0.0371	0.0329	0.0371	0.0076	0.0253	0.1862	0.1730	0.0132	0.0112
Mean	0.0285	0.0054	0.0700	0.0403	0.0297	0.0385	0.0012	0.0308	0.1848	0.1730	0.0118	0.0112
Residual solution	—	—	0.0728	0.0366	0.0362	0.0385	0.0110	0.0233	0.1862	0.1728	0.0134	0.0126
Stage: 15 days												
(1)	0.0251	0.0052	0.0641	0.0293	0.0351	0.0343	0.0141	0.0160	0.1666	0.1512	0.0154	0.0098
(2)	0.0251	0.0052	0.0658	0.0288	0.0370	0.0336	0.0177	0.0145	0.0166	0.1502	0.0164	0.0084
Mean	0.0251	0.0052	0.0651	0.0290	0.0361	0.0340	0.0159	0.0152	0.0166	0.1507	0.0159	0.0091
Residual solution	—	—	0.0630	0.0307	0.0323	0.0343	0.0099	0.0188	0.0166	0.1517	0.0149	0.0084
Stage: hatched chick												
(1)	0.0216	0.0045	0.0658	0.0298	0.0360	0.0357	0.0138	0.0163	0.1568	0.1360	0.0208	0.0084
(2)	0.0214	0.0042	0.0630	0.0333	0.0297	0.0357	0.0044	0.0229	0.1540	0.1385	0.0155	0.0070
Mean	0.0215	0.0043	0.0644	0.0315	0.0329	0.0357	0.0092	0.0175	0.1554	0.1373	0.0181	0.0077
Residual solution	—	—	0.0658	0.0290	0.0368	0.0357	0.0150	0.0151	0.1540	0.1353	0.0187	0.0084

The sulphur estimations, in terms of cystine N, were:

	Cystine N			
	Exp. 1		Exp. 2	
	Diamino	Monoamino	Diamino	Monoamino
Stage: 0	0.0084	0.0161	0.0035	0.0114
" 15 days	0.0082	0.0159	0.0031	0.0076
" hatched chick	0.0126	0.0147	0.0014	0.0082

The amounts of bromine absorbed, and the corresponding amounts of tyrosine nitrogen, were:

	Exp. 1		Exp. 2	
	Br	Tyrosine N	Br	Tyrosine N
Stage: 0	0.516	0.0225	0.304	0.0132
" 15 days	0.466	0.0203	0.278	0.0121
" hatched chick	0.429	0.0187	0.224	0.0098

Expressed in terms of percentage of the total nitrogen the data come out as follows.

The two sets of figures giving a mean are from the mean result of the several analyses and from the result using the combined solutions which remained from the separate determinations.

	Exp. 1			Exp. 2		
	Stage			Stage		
	0	15 days	hatched	0	15 days	hatched
Amide	8.2	8.7	8.2	9.8	9.5	8.7
Humin	1.5	1.8	1.7	1.8	1.9	1.8
Diamino:						
Total	25.7 } 25.8	26.7 } 27.1	27.0 } 27.4	24.0 } 24.5	24.6 } 24.2	26.1 } 26.4
Amino	14.9 } 14.8	15.5 } 15.0	14.7 } 14.5	13.8 } 13.2	11.0 } 11.3	12.8 } 12.2
Non-amino	10.8 } 11.0	11.1 } 11.9	12.3 } 13.0	10.2 } 11.3	13.6 } 12.9	13.3 } 14.1
Arginine	13.9 } 14.3	14.6 } 15.0	16.6 } 16.8	13.2 } 13.2	13.5 } 13.5	14.5 } 14.5
Histidine	0.5 } 0.4	0.3 } 1.2	0.1 } 0.5	0.4 } 2.1	6.0 } 4.8	3.8 } 4.9
Lysine	11.2 } 11.0	11.7 } 10.9	10.6 } 10.2	10.4 } 9.2	5.7 } 6.4	7.1 } 6.6
Cystine	10.9 } 1.8	10.1 } 1.7	9.5 } 2.6	8.0 } 1.2	7.1 } 1.2	6.1 } 0.6
Monoamino:						
Total	64.1 } 64.0	61.0 } 61.3	60.5 } 60.2	63.4 } 63.7	62.9 } 62.9	63.1 } 62.8
Amino	63.9 } 63.4	61.6 } 60.0	60.0 } 56.7	63.9 } 59.3	62.9 } 57.1	62.5 } 55.3
Non-amino	0.6	1.3	4.5	59.4 } 4.3	56.9 } 5.8	55.8 } 7.4
Arginine	4.6 } 4.4	3.7 } 3.6	3.3 } 3.2	4.1 } 4.0	6.0 } 3.1	7.3 } 3.3
Cystine	4.3 } 3.5	3.4 } 4.9	3.2 } 5.7	4.3 } 3.9	3.2 } —	3.4 } 3.3

#### DISCUSSION OF RESULTS.

No change can be considered to occur in the amide and humin nitrogen, though a small decrease was observed in the amide nitrogen in the second experiment.

In both experiments a distinct increase occurred in the total diamino-nitrogen amounting to about 2 % of the total nitrogen of the egg proteins. Corresponding with this increase there was an increase in the amount of arginine nitrogen amounting to about 1 %. The figure in the first experiment with the hatched chick is probably high from error in the amide determination.



As has been previously experienced the estimation of the amino-nitrogen has given very erratic results; consequently no stress can be laid upon the figures for histidine and lysine; they probably increase, as the arginine increase only accounts for half the increase of the total diamino-nitrogen.

The total monoamino-nitrogen definitely decreased in both experiments. In the first experiment the amino-nitrogen figures were too high, but on repetition at a later date on the combined residues, they showed a decrease, as found in the second experiment. There was an increase in the non-amino-nitrogen.

Cystine nitrogen increased in both the diamino- and monoamino-fractions in the first experiment; in the second experiment the amounts of barium sulphate were so small that the figures are probably not correct.

The bromine absorption figures indicate a marked decrease during development. As the cystine nitrogen increases, this change probably indicates a decrease in the tyrosine content.

It would thus appear that in the metabolism of the developing egg the monoamino-acids are used for the formation of urea, uric acid, etc., and for furnishing energy, whilst the diamino-acids are retained for building the body protein of the chick. Loss of monoamino-acid would leave a remainder with a higher diamino-acid content. The changes run parallel with the changes which have been observed with salmon during the spawning season. These fish derive their energy from the monoamino-acids of the muscular tissue, whilst the diamino-acids pass to the reproductive organs. The eggs of the brook-trout and salamander have also been found [see Needham, 1925] to show an increase of diamino-nitrogen during development.

#### SUMMARY.

Estimation of the amino-acids in the hen's egg during development by the modified Van Slyke method has shown that there is an increase of about 2 % in the diamino-nitrogen, of which the arginine increase makes about 1 % of the total nitrogen of the egg proteins. The monoamino-acid nitrogen decreases by about 4 % during development with a corresponding decrease in the amino-nitrogen content.

We gratefully acknowledge a grant to one of us from the Government Grant Committee of the Royal Society for defraying the expenses of the work.

#### REFERENCES.

- Fiske and Boyden (1926). *J. Biol. Chem.* **70**, 535.
- Needham (1925). *Physiol. Reviews*, **5**, No. 1.
- (1926, 1). *Brit. J. Exp. Biol.* **3**, 189.
- (1926, 2). *Brit. J. Exp. Biol.* **4**, 114, 145.
- Plimmer and Lowndes (1927). *Biochem. J.* **21**, 247.
- Plimmer and Phillips (1924). *Biochem. J.* **18**, 312.
- Plimmer and Rosedale (1925). *Biochem. J.* **19**, 1015.
- Plimmer and Scott (1909). *J. Physiol.* **38**, 247.
- Sendju (1925). *Japan. J. Biochem.* **5**, 391.

## XXXVI. A NOTE ON THE ESTIMATION OF CHLORINE IN MILK.

BY ALFRED DUDLEY HUSBAND AND WILLIAM GODDEN.

*From the Rowett Research Institute, Aberdeen.*

*(Received November 11th, 1926.)*

AUSTIN AND VAN SLYKE [1920] have shown that the Van Slyke-Donleavy [1919] method for the determination of plasma chlorides, when applied to whole blood, gives results too high by 30 to 40 %. If, however, the protein is precipitated by picric and nitric acids, and the protein-free filtrate is treated with silver nitrate, a quantitative precipitation of the total chloride of the whole blood is obtained and the final titration may be carried out as in the Van Slyke-Donleavy method.

Sisson and Dennis [1921], employing the Van Slyke-Donleavy method, with slight modifications, for the determination of chlorine in human milk, claim to have obtained results essentially similar to those obtained by fusion of the milk with sodium carbonate, to remove the organic material, and subsequent titration by the Volhard method. We have investigated the suitability of these two methods for the determination of chlorine in milk and have checked our results by means of Volhard titration after ashing the milk with sodium carbonate or with lime.

It was found, in testing these methods, that it was advisable to use the modified picric acid solution suggested by Sisson-Dennis to obtain complete precipitation of the milk proteins. The technique as used by us was as follows.

(a) *Method of Sisson-Dennis.* To 10 cc. of milk measured into a small flask were added 20 cc. of 1.2 % picric acid solution containing 2 cc. of glacial acetic acid per litre. The mixture was well shaken and allowed to stand for 5-10 minutes. 10 cc. of  $N/10$  silver nitrate were then added, the mixture was again shaken and filtered on a chlorine-free filter paper. 20 cc. of clear filtrate were measured off and titrated against standard ammonium thiocyanate solution, as in the Volhard method.

(b) *Method of Austin and Van Slyke.* To 20 cc. of milk measured into a small flask were added 40 cc. of the picric acid solution, and after 10 minutes the curd was filtered off on a chlorine-free filter paper. 30 cc. of clear filtrate were then treated with 10 cc. of  $N/10$  silver nitrate, the mixture was shaken, again filtered and 20 cc. of the clear filtrate were titrated against the standard thiocyanate.

The Volhard method for making the final titration was adopted for the sake of convenience.

The results obtained by us do not agree with the findings of Sisson-Dennis, but are in entire agreement with the results obtained by Austin and Van Slyke on whole blood.

In all the milks we tested the figures obtained were too high unless the protein was first removed before adding the silver nitrate solution. A comparison of the percentage of chlorine found by the different methods is shown in Table I.

Table I.

No. of sample	Animal	Method of Sisson-Dennis Cl %	Method of Austin-Van Slyke Cl %	Method of ashing Cl %
1	Cow	0.1276	0.1028	0.1028
2	"	0.1276	0.1064	—
3	"	0.1347	0.1064	—
4	Goat	0.1808	0.1560	—
5	"	0.1454	0.1135	0.1135

The figures given in Table II indicate that when using the method of Sisson-Dennis the results are liable to vary if changes are made in the ratio of milk to silver nitrate used in the determination, whereas, by the method of Austin-Van Slyke, the percentage of chlorine found is independent of these changes provided that an excess of silver nitrate is present.

Table II. *Effect of varying the quantities of milk and silver nitrate.*

No. of sample	Amount of milk cc.	Amount of AgNO <sub>3</sub> cc. N/10	Method of Sisson-Dennis Cl %	Method of Austin-Van Slyke Cl %
1	10	10	0.1276	0.1064
1	20	10	0.1383	—
1	10	20	0.1312	—
2	10	10	0.1347	0.1064
2	5	10	—	0.1090
2	20	10	—	0.1064

The only effect of varying the amount of picric acid is to change the rate of filtration. In view of the fact that the presence of the protein of milk gives values too high when determining the chlorine content of milk a few tests were carried out on a chlorine-free solution of caseinogen using the methods described above.

Table III shows that only by the method of Sisson-Dennis was any apparent chlorine content found. This would appear to be due to either adsorption or combination of some of the silver by the precipitated protein.

Table III. *Test of methods on chlorine-free solution of caseinogen.*

Amount of caseinogen in solution g.	Apparent Cl-content found by method of Sisson-Dennis g.	Cl found by method of Van Slyke	Cl found by ashing with lime
0.3	0.00354	Nil	Nil
0.3	0.00213	"	"

## CONCLUSION.

To determine accurately the chlorine content of milk volumetrically by precipitation of the chlorine as silver chloride, it is essential that the protein be removed before the addition of the silver nitrate.

## REFERENCES.

- Austin and Van Slyke (1920). *J. Biol. Chem.* **41**, 345.  
Sisson and Dennis (1921). *Amer. J. Dis. Child.* **21**, 389.  
Van Slyke and Donleavy (1919). *J. Biol. Chem.* **37**, 551.

# XXXVII. THE BACTERIAL DECOMPOSITION OF TOBACCO AS LEADING TO THE FORMATION OF BASES IN THE PRESENCE OF WATER.

By A. FAITELOWITZ.

(Received November 15th, 1926.)

IN work published in this *Journal* Fodor and Reifenberg [1925] came to the following conclusions.

(1) Tobacco extracts are capable of decomposing nicotine into pyridine and amino-bases, during which process a gradual increase in alkalinity takes place.

(2) The nicotine-decomposing activity of fermented tobacco is found to be smaller than that of non-fermented tobacco.

(3) Tobacco leaves dried artificially and quickly at 30° proved to be less active in decomposing nicotine than leaves dried slowly and naturally.

(4) The well-known amelioration of the quality of tobacco due to fermentation is attributed by Fodor and Reifenberg to the decomposition of a part of the nicotine through the action of an oxidase into pyridine and amino-bases. They consider that the aromatic qualities of tobacco are thus affected while denying the formation of acids during that process.

Numerous experiments are quoted with tobacco of different brands and origin which, however, appear to be hardly comparable among themselves, thus leaving the last three conclusions open to objection.

The first finding was confirmed by the writer, though this fact had to be differently interpreted in view of other discordant results.

For instance, the important rôle attributed by Fodor and Reifenberg to the decomposition of part of the nicotine into pyridine and amine with development of alkalinity is, in fact, quite insignificant for tobacco fermentation as demonstrated in the following preliminary qualitative tests. These tests rather indicate that the splitting up of nicotine and the subsequent increase in alkalinity gradually impair the quality of tobacco.

## EXPERIMENTAL.

(a) To 10 g. of cigarette tobacco 90 cc. of water were added. After leaving the mixture to stand for 2 hours the macerate was separated from the tobacco by squeezing the latter in a cloth. All macerates so treated gave without exception an acid reaction to litmus paper. The acidity of macerates of

different brands varied from 15–20 cc. *N*/10 NaOH for 100 cc. of tobacco macerate.

(b) If a tobacco macerate is left to stand for a long time at room temperature (17–20°)—no matter whether filtered or not—then the acidity gradually begins to fall off and the macerate turns alkaline after about 30 hours' standing.

(c) On addition of 2 cc. chloroform to 10 cc. of a still acid tobacco macerate, kept in a well-stoppered bottle in order to prevent evaporation of chloroform, the reaction remains acid, while in another stoppered bottle the same macerate without addition of chloroform turns alkaline after a certain length of time.

From the foregoing experiments (a), (b) and (c) we learn that fermented cigarette tobacco (for cigarettes consist exclusively of fermented tobacco) does not become alkaline during the dry fermentation process, but does so if brought into contact with water. Such basic reaction should be attributed to bacterial action rather than to oxidase, for it is a well-established fact that chloroform does not interfere with enzymic reaction but arrests bacterial activity [Faitelowitz, 1910].

(d) Treatment of cigarette tobacco:

40 cc. of water are added to 10 g. of cigarette tobacco. The mixture is kept in a covered vessel at room temperature till the reaction turns basic. Then the pulp is spread out in a wide open dish and left to stand for some time (2–3 days) till the macerate shrinks as the water evaporates. It is then left until a perfectly air dry condition is reached. Tobacco so treated becomes black, gives a basic reaction to litmus paper and evolves an odour probably due to pyridine and amino-bases. The tobacco is then unpleasant when smoked and has no value as cigarette tobacco.

As regards the dry curing process it will be the object of a further detailed study to determine the rate of development of acidity and catalase content in freshly cut tobacco leaves, in leaves dried slowly but not yet fermented, and in already fermented leaves. Should an increase of catalase and acidity be found in fermented leaves, then it would become apparent that tobacco fermentation is a similar process to milk fermentation where acid-forming bacteria produce lactic acid and catalase [see also Isajeff, 1904].

#### SUMMARY.

1. The formation of pyridine and amino-bases from nicotine with a resulting basic reaction does not occur to any appreciable extent in the dry fermentation process.

2. In the presence of moisture the decomposition of tobacco leads to a basic reaction, but this condition reduces the quality of tobacco for the purpose of smoking.

3. The decomposition of the tobacco leaf in the presence of water is not due to enzymic action but most probably to the action of bacteria, since, in the presence of chloroform which eliminates bacterial action, the reaction of tobacco remains permanently acid.

## REFERENCES.

- Faitelowitz (1910). *Zentr. Milchwirt.* Nos. 7, 8 and 9.  
Fodor and Reifenberg (1925). *Biochem. J.* **19**, 830.  
Isajeff (1904). *Z. physiol. Chem.* **42**, 102.

## OBITUARY NOTICE.

### JOHN WEBSTER.

JOHN WEBSTER, who died on January 20th from sudden heart failure following influenza, was a member of an old Quaker family, of Birmingham, where he was educated at Mason's College. He was a Fellow of the Chemical Society, of the Society of Public Analysts (of whose Council he was at one time a member), and of the Royal Society of Medicine. He was a member of the Medico-Legal Society, and of its council at the time of his death, and was also a member of the Biochemical Society.

Mr Webster was a most experienced toxicological analyst. About 1900 he became assistant analyst to Sir Thomas Stevenson, who was then senior scientific analyst to the Home Office, and remained in association with him until his death in 1908. Sir William Willcox succeeded Sir Thomas Stevenson, and Mr Webster became assistant analyst to him; and in 1919, when Sir William Willcox retired, Mr Webster was appointed senior scientific analyst to the Home Office.

Mr Webster was an analyst in whom absolute reliance could be placed as regards accuracy, which was essential in work connected with poisoning cases where the life of an accused person might depend on the result of an analysis. His ability was shown in many of the most famous poisoning cases in recent criminal history; he was engaged in the scientific investigations connected with the trials of Seddon, Greenwood, Armstrong, Bywaters and Mrs Thompson, and Vacquier, as well as in a number of smaller investigations.

During the past twenty years he had been on the staff of the Pathological Chemistry Department of St Mary's Hospital, Paddington, and had held the position of pathological chemist to the Hospital.

His great analytical skill and experience were of the utmost value in the clinical investigation of the pathological problems associated with cases in the wards of the Hospital, and they furnished a reliable guide in diagnosis and treatment of many of the difficult cases.

Mr Webster was a most painstaking, careful and neat investigator. He would be seen at his best when carrying out the toxicological investigation of viscera where great difficulties were encountered in consequence of putrefactive changes. The extraction of chemical poisons such as vegetable alkaloids in such cases requires the utmost skill and experience, and at work of this kind he showed a masterly ability and a brilliant technique which had been acquired by long experience and meticulous care.



He would always be absolutely sure of the accuracy of his results and if necessary confirm them by repeated analyses.

His work on the excretion of arsenic after the administration of either arsenic by the mouth or an arseno-benzene derivative intravenously has been of the utmost value not only in toxicological investigations but in guidance as to the correct time for the administration of repeated doses of arseno-benzene compounds.

In the recent Armstrong case the amount of arsenic found by Mr Webster in urine passed three days after the taking of the poison enabled him to say that the amount administered three days previously was a possible fatal dose. He published several papers on research work, especially with reference to arsenical poisoning and the toxicology of salvarsan.

During the War Mr Webster's time was fully occupied by his toxicological work for the Home Office and his Pathological Chemical work at St Mary's Hospital. He did not work on trinitrotoluene poisoning. The "Webster Test" of the urine in this condition was discovered by Mr T. A. Webster working in conjunction with Dr H. H. Dale and Dr Barger<sup>1</sup>. The "Webster Test" has been, in a recent obituary notice, incorrectly attributed to the late Mr J. Webster, F.I.C., F.C.S.

During the past twenty years, while at St Mary's Hospital, Mr Webster had closely identified himself with the work of the Hospital and Medical School. He was ever ready to help in the teaching of Forensic Medicine and Chemical Pathology, and whenever his great experience was required in any analytical investigation he was always most willing to help any student in difficulties.

He was a Vice-President of the Students' Musical Society at St Mary's Hospital and had conducted Concerts for them. He also had been for some years a member of the Sancta Maria Lodge and during that time had held the post of Honorary Organist.

His kindly disposition had endeared him to many of his friends and colleagues at St Mary's Hospital, and he will be much missed by them. His death will be a great loss to toxicological science.

He leaves a widow and one son.

<sup>1</sup> Medical Research Council Special Report Series, No. 58, *TNT poisoning and the fate of TNT in the animal body*.

W. H. WILLCOX.

# XXXVIII. THE USE OF THE HYDROQUINHYDRONE ELECTRODE FOR $p_H$ DETERMINATION IN THE FLUIDS OF THE ORGANISM.

By FELIX GROSSMAN.

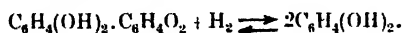
*From the Physiological Laboratory, The University of Wilno, Poland.*

*(Received November 26th, 1926.)*

In the beginning of the year 1925 I began the investigation of the applicability of Biilmann's [1921] quinhydrone electrode to determine the  $p_H$  of serum, cerebrospinal fluid and blood. There has since appeared a number of papers showing that there is a divergence of opinion as to the suitability of this electrode for these purposes. The different experiences have recently been reviewed by Mislowitzer [1926] who adheres to his opinion, expressed earlier [1925], that the quinhydrone electrode can be employed to determine the  $p_H$  of blood.

My first measurements with a quinhydrone electrode gave unsatisfactory results. The potential was quite unstable. By diluting the serum several times with physiological saline solution I obtained somewhat better results. However, having made several measurements which did not give consistent results, I gave up the quinhydrone electrode and tried the hydroquinhydrone electrode, also invented by Biilmann [1921]. The difference between the quinhydrone and the hydroquinhydrone electrodes is that, instead of quinhydrone, an excess of a quinol (hydroquinone) and quinhydrone mixture is used. Biilmann and Lund [1921] recommend 0.1 g. of quinhydrone and 1 g. of quinol for 15 cc. of fluid. Part of this mixture is dissolved, the rest remains as a precipitate. The platinum wire of the electrode must be almost completely immersed in the precipitate.

The reaction in such an electrode consists in the transformation of stable quinhydrone into stable quinol:



The electromotive force of the chain: Pt/quinol, quinhydrone, solution A/KCl/solution A,  $H_2$ /Pt, according to Biilmann and Lund [1921] = 0.6179 volts at  $t = 18^\circ$ , instead of 0.7042 in the case of the quinhydrone electrode.

With serum the potential became rapidly stable and the values obtained for  $p_H$  were consistent. They differed, however, from those obtained with the hydrogen electrode (see Table I).

Table I.

*Pig-serum.* $t = 22^\circ$ .

No.	Hydrogen electrode				Hydroquinhydrone electrode					
	Volts	Average	$p_H$	Average	Volts	Average	$p_H$	Average	$\Delta p_H$	$p'_H$
1	0.708*		7.93		-0.068†		7.67			
2	0.707	0.7075	7.91	7.92	-0.070	-0.0687	7.70	7.68	0.19	7.87
3					-0.068		7.68			

*Ox-serum.* $t = 18^\circ$ .

1	0.691		7.76		-0.066		7.63			
2	0.692	0.693	7.78	7.79	-0.067	-0.0667	7.65	7.64	0.18	7.82
3	0.695		7.83		-0.067		7.65			

\* These values are for chain: Pt/H<sub>2</sub>, serum/KCl/satur. KCl, Hg<sub>2</sub>Cl<sub>2</sub>/Hg.† These values are for chain: Pt/quinol, quinhydrone, serum/KCl/satur. KCl, Hg<sub>2</sub>Cl<sub>2</sub>/Hg.

After satisfying myself that my determinations of the  $p_H$  of pig-serum with the hydrogen electrode were correct, I repeated the measurements on ox-serum with both electrodes. I again obtained with the hydroquinhydrone electrode a similar difference from those obtained with the hydrogen electrode. Having previously tested this electrode upon standard solutions with  $p_H < 7$  and found it to be very exact, I proceeded to ascertain its behaviour with slightly alkaline but well buffered solutions.

For this purpose I prepared a series of Sørensen's phosphate solutions and determined their  $p_H$  values with a hydrogen, a quinhydrone and a hydroquinhydrone electrode respectively (see Table II).

I obtained, as seen from Table II, differences between  $p_H$  determined with the hydroquinhydrone electrode and the quinhydrone electrode, increasing as the fluid became more alkaline.

Upon the ground of these determinations I arrived at an empirical formula for the correction to be applied when using the hydroquinhydrone electrode on a fluid with  $p_H > 7$ , namely,  $\Delta p_H = 0.24 \times 2^{p_H - 7} - 0.21$ .

If we take into consideration  $p_H = 7.00$ – $8.00$ , that is the widest limits in which  $p_H$  can balance in the serum, blood, or cerebrospinal fluid of men and animals, then the rectification  $\Delta p_H$  will vary from 0.03 to 0.21. For body fluids  $\Delta p_H$  will not on the average exceed 0.13.

These corrections are introduced in the tables, and it will be seen that the values with the hydrogen and the hydroquinhydrone electrodes then become almost identical.

I am now working on the theoretical confirmation of the rectification,  $\Delta p_H$ , introduced by me, as was done by La Mer and Parsons [1923] for the aberrations of the quinhydrone electrode in alkaline solutions.

A hydroquinhydrone electrode possesses many advantages over a hydrogen electrode for determining the  $p_H$  in serum, blood, etc., both as to simplicity and rapidity in making determinations. Further, there are many conditions under which the hydrogen electrode cannot be used at all.

Table II.

<i>t</i> = 18°.										
No.	Hydrogen electrode			Quinhydrone electrode			Hydroquinhydrone electrode			
	Volts	Average	<i>p</i> <sub>H</sub> average	Volts	Average	<i>p</i> <sub>H</sub> average	Volts	Average	<i>p</i> <sub>H</sub> average	$\Delta p_{\text{H}}$ average
Mixture of equal volumes of <i>M</i> /15 Na <sub>2</sub> HPO <sub>4</sub> and <i>M</i> /15 KH <sub>2</sub> PO <sub>4</sub> ; according to Sørensen <i>p</i> <sub>H</sub> = 6.80.										
1	0.634			0.0680			-0.0180			
2	0.636	0.6347	6.79	0.0680	0.0680	6.81	-0.0185	-0.0182	6.81	0.01
3	0.634			0.0680			-0.0180			
Mixture of 61 volumes of Na <sub>2</sub> HPO <sub>4</sub> and 39 volumes of KH <sub>2</sub> PO <sub>4</sub> , with <i>p</i> <sub>H</sub> = 7.00.										
<i>t</i> = 18°.										
1	0.646			0.0570			-0.0260			
2	0.648	0.647	7.00	0.0570	0.0570	7.00	-0.0270	-0.0267	6.97	0.03
3	0.647			0.0570			-0.0270			
Mixture of 80 vol. of Na <sub>2</sub> HPO <sub>4</sub> and 20 vol. of KH <sub>2</sub> PO <sub>4</sub> , with <i>p</i> <sub>H</sub> = 7.36.										
<i>t</i> = 19°.										
1	0.669			0.0345			-0.0460			
2	0.671	0.670	7.38	0.0345	0.0347	7.38	-0.0465	-0.0465	7.28	0.10
3				0.0350			-0.0470			
Mixture of 87 vol. of Na <sub>2</sub> HPO <sub>4</sub> and 13 vol. of KH <sub>2</sub> PO <sub>4</sub> , with <i>p</i> <sub>H</sub> = 7.60.										
<i>t</i> = 19°.										
1	0.683			0.0230			-0.0555			
2	0.680	0.681	7.56	0.0230	0.0232	7.56	-0.0560	-0.0557	7.43	0.13
3	0.680			0.0235			-0.0555			
Mixture of 60 vol. of borate and 40 vol. of 0.1 <i>N</i> HCl, with <i>p</i> <sub>H</sub> = 8.27										
<i>t</i> = 18°.										
1	0.719			-0.0150			-0.0850			
2	0.720	0.719	8.25	-0.0160	-0.0155	8.26	-0.0850	-0.0855	7.98	0.275
3	0.718			-0.0155			-0.0855			
Mixture of 84 vol. of borate and 16 vol. of 0.1 <i>N</i> HCl, with <i>p</i> <sub>H</sub> = 8.99.										
<i>t</i> = 18°.										
1	0.760			-0.0370			-0.1150			
2	0.762	0.7607	8.97	-0.0360	-0.0360	8.96	-0.1150	-0.1145	8.49	0.475
3	0.760			-0.0350			-0.1135			

I, therefore, made parallel determinations of the *p*<sub>H</sub> of serum, blood and cerebrospinal fluid with the hydroquinhydrone and hydrogen electrodes. These are set forth in Table III.

We see that after introducing the correction for the hydroquinhydrone electrode, the results obtained with this electrode and those obtained with the hydrogen electrode, given in Table III, are almost identical. I made measurements in the following way: I poured from 1.5 to 3 cc. of physiological salt solution into a small electrode vessel and added a mixture of dry quinhydrone and quinol in quantities more or less such as I have mentioned above; I stirred the contents of the small vessel, poured in 1.5 cc. of blood—that is, diluted it from 1 : 1 to 1 : 2—stirred the contents of the vessel well and after about three minutes read the potential of this electrode. The more the blood is diluted, the better and the more rapidly the potential of the electrode becomes

stable. The relative amounts of quinhydrone and quinol need not necessarily be exactly those recommended by Büllmann. After one has made several measurements, one can judge sufficiently correctly by eye, but the greatest part of the short platinum wire must be immersed in the precipitate.

Table III.

No.	Hydrogen electrode			Hydroquinhydrone electrode				
	Volts	Temp.	$p_H$	Volts	Temp.	$p_H$	$\Delta p_H$	$p'_H$
<i>Human serum.</i>								
1	0.6855	20	7.63	-0.0625	20	7.51	14.5	7.655
2	0.6820	18	7.61	-0.0580	18	7.50	14.5	7.645
3	0.6810	18	7.59	-0.0550	18	7.45	13	7.58
4	0.6820	20	7.56	-0.0560	20	7.40	12	7.52
5	0.6820	19	7.59	-0.0560	19	7.43	13	7.56
<i>Cerebrospinal fluid.</i>								
1	0.6910	20	7.72	-0.0640	20	7.53	15	7.68
2	0.6900	20	7.70	-0.0625	19.5	7.51	15	7.66
3	0.6830	20	7.58	-0.0615	20	7.48	14	7.62
4	0.6840	18	7.64	-0.0550	18	7.45	13	7.58
5	0.6850	18.5	7.65	-0.0580	18	7.50	14.5	7.645
<i>Rabbit blood.</i>								
1	0.678	17.5	7.55	-0.050	17.5	7.38	0.12	7.50
2	0.671	18	7.42	-0.048	18	7.33	0.11	7.44
3	0.6805	17.5	7.59	-0.054	17.5	7.45	0.13	7.57
4	0.673	17	7.48	-0.046	16.5	7.35	0.115	7.465
5	0.683	18.5	7.62	-0.056	18.5	7.46	0.13	7.60
<i>Human blood.</i>								
1	0.669	18	7.39	-0.045	18	7.28	0.10	7.38
2	0.675	18.5	7.47	-0.047	18	7.31	0.11	7.42
3	0.668	18	7.37	-0.043	18	7.25	0.10	7.35
4	0.674	18	7.45	-0.050	18.5	7.34	0.115	7.455
5	0.678	18.5	7.53	-0.053	18	7.42	0.125	7.545

Making several measurements on the same blood, for instance that of the rabbit, diluted with 0.85 % NaCl solution, I obtained with the hydroquinhydrone electrode almost identical values, for instance, 7.52, 7.52, 7.54. The quinhydrone electrode in the case of serum as in that of blood gave improbable and drifting results.

I attempted too, to use with blood the quinoquinhydrone electrode also invented by Büllmann [1921] for which a mixture of quinone and quinhydrone is employed. The reaction consists in the transformation of stable quinone into stable quinhydrone:



This electrode is unsuitable for blood as it has a very drifting potential. It is also inferior to the hydroquinhydrone electrode for serum.

I have found that the quinhydrone, hydroquinhydrone and especially the quinoquinhydrone electrode, as pointed out by La Mer and Parsons, cannot be used for fluids with  $p_H > 9$ , because the quinol oxidises too rapidly.

I have tried the gold electrode recommended by Dixon and Quastel [1923], Corran and Lewis [1924] and Mislowitzer [1925] but have not found it satisfactory. Mislowitzer [1926] has also abandoned it in favour of platinum.

#### SUMMARY.

To determine the  $p_H$  in physiological fluids such as, for instance, serum, plasma, blood, cerebrospinal fluid, etc., the hydroquinhydrone electrode is more exact and simpler than the hydrogen electrode and more generally applicable and, if a correction varying with the alkalinity of the solution is applied, affords results which agree with those obtained with the hydrogen electrode.

#### REFERENCES.

- Biilmann (1921). *Ann. Chim.* **15**, 109.  
Biilmann and Lund (1921). *Ann. Chim.* **16**, 321.  
Corran and Lewis (1924). *Biochem. J.* **18**, 1358.  
Dixon and Quastel (1923). *J. Chem. Soc.* **123**, 2943.  
La Mer and Parsons (1923). *J. Biol. Chem.* **57**, 613.  
Mislowitzer (1925). *Biochem. Z.* **159**, 77.  
—— (1926). *Klin. Woch.* 1863.

## XXXIX. PHOSPHORYLATION OF PROTEINS.

By CLAUDE RIMINGTON.

*(Benn W. Levy Student, Research Scholar of Emmanuel College, Cambridge.)*

*From the Carlsberg Laboratory, Copenhagen, and the Biochemical Laboratory, Cambridge.*

*(Received December 31st, 1926.)*

THE peculiarity of the phosphoproteins in containing relatively large quantities of phosphorus, organically combined, and the unique importance of these substances in the nutrition of the young organism has led in the past to a good deal of speculation as to the mode of combination by which the phosphorus is held in the molecule, and also to attempts on the part of one or two workers to produce similar phosphorus-containing bodies by the action of various reagents upon proteins.

Liebermann [1888] and Pohl [1889] investigated the action of metaphosphoric acid upon proteins. Phosphorus oxychloride appears to have been used first by Bechhold [1901] who treated egg-albumin, dissolved in aqueous alkaline solution, with this reagent. There was, however, great development of heat during the addition of the phosphorus oxychloride and a consequent destruction of the protein. The resulting reaction-mixture was found to contain substances, some of which were precipitable by acid, which contained phosphorus, and exhibited properties characterising them as derived proteins resembling albumoses in complexity.

□ A modification of the method was attempted by Neuberg and Pollak [1910] and later by Neuberg and Oertel [1914], the phosphorus oxychloride being dissolved in chloroform. Lactalbumin, serum globulin and caseinogen were "phosphorised" in this way and the phosphorised products precipitated by acetic acid and washed until no free acid remained in the filtrate. They contained amounts of phosphorus varying from 1.28 % in lactalbumin to 1.77 % in serum globulin. These authors also made some experiments of a purely qualitative nature upon the behaviour of the compounds towards the digestive enzymes. Neuberg and Oertel [1914] also state that phosphorised globulin is coagulated by rennin, an observation which the present author has not been able to confirm, as in his experience the calcium salt of the phosphorised protein is already insoluble under the conditions in which the test would be carried out.

Undoubtedly the technique employed by Neuberg affords a method of bringing about organic combination of phosphorus with the protein, but no

conclusive evidence was brought forward that the products were free from admixture with inorganic phosphoric acid. The difficulty of removing small quantities of this substance from protein precipitates is very great, and it seems that a couple of reprecipitations, even accompanied by thorough washing, is insufficient to secure complete freedom from inorganic impurities.

In the present work, a method is used by which the progressive purification of the product is followed by determining the P/N ratio of the *filtrates* from each reprecipitation. The process of reprecipitation and washing is repeated until the P/N ratio of the filtrate remains constant and is then equal, it is found, to the P/N ratio of the precipitated substance itself. By this procedure absolute certainty is achieved that all the phosphorus remaining in the product is in definite chemical union, while at the same time none of the substance itself is used up in analyses during its purification—a point of undoubted advantage when small quantities of material are being dealt with.

The method of carrying out the phosphorylation is analogous to the Schotten-Baumann reaction for the benzylation of organic substances in aqueous solution. By this means the high concentrations of alkali employed by Neuberg are avoided and the risk of destructive changes in the proteins thereby diminished, for it has already been shown by Rimington and Kay [1926] that under the action of 1 % sodium hydroxide at 37° caseinogen rapidly loses both phosphorus (as phosphoric acid) and ammonia.

The proteins used in this investigation are caseinogen, serum globulin, prepared from horse serum, carefully purified and subsequently denatured by alcohol, and “dephosphorised caseinogen,” the phosphorus-free protein obtained by the action of 1 % sodium hydroxide for 24 hours upon caseinogen at 37°. The globulin was chosen for the following reasons. It was shown by Crowther and Raistrick [1916] and confirmed by Woodman [1921] that the globulins of serum, colostrum and of milk are identical, whilst the albumin of serum differs from that present in colostrum and milk. The proportion of lactoglobulin in colostrum is very high, 6–12 %, and falls immediately to the low figure of 0.005 % as soon as secretion of true milk commences.

In considering the problem of mammary secretion, the possibility has always to be kept in mind that one or other of the blood proteins might conceivably furnish the starting point for the synthesis of caseinogen. The ready permeability of the cells of the mammary gland to serum globulin, as evidenced by the identity of the protein in serum, colostrum and milk respectively, taken together with the difference in the amounts occurring in the two secretions makes this possibility worthy of further consideration. Moreover the distribution of nitrogen in caseinogen and in lactoglobulin shows some similarity as will be seen from the figures given by Crowther and Raistrick [1916].

It was felt that phosphorylation of serum globulin would be of interest not only in so far as it helped to throw light upon the manner in which the synthetically introduced phosphorus atoms are held in combination with the protein but also with reference to the problem of the origin of caseinogen in



the mammary cell. The stability of the phosphorus in phosphorised globulin towards acids, alkalis and enzyme preparations has been investigated upon lines similar to those adopted by Rimington and Kay in the study of caseinogen, and a striking similarity has been found to exist between these two proteins, suggesting a similar type of linkage. It is hoped that the accumulation of additional facts will afford evidence, in the light of which the hypothesis outlined above may be examined and a clearer knowledge obtained of the materials entering into the synthesis of caseinogen *in vivo*.

#### PHOSPHORYLATION OF PROTEINS.

In all cases the proteins were first thoroughly purified by solution and reprecipitation at the isoelectric point several times in succession. Caseinogen was directly prepared from separated milk. Care was taken to avoid undue exposure to alkali, the sodium hydroxide being added drop by drop, over a period of several hours to the suspension of protein in water which was vigorously agitated by a mechanical stirrer. A quantity of the protein, between 7 and 10 g., was then dissolved in water with the aid of the requisite quantity of sodium hydroxide, the total volume being brought up to 125–150 cc.

Phosphorus oxychloride (20–25 g.) dissolved in eight times its volume of carbon tetrachloride was allowed to drop into the ice-cold, rapidly stirred protein solution, to which had been added phenolphthalein to serve as an indicator, and the reaction was kept faintly alkaline by addition of 4*N* sodium hydroxide. The addition of the oxychloride extended over 6–8 hours and the temperature of the reactants never rose above 5°.

After separating the aqueous layer from the carbon tetrachloride, the phosphorised protein could be precipitated by addition of 2*N* hydrochloric acid until maximum separation occurred. This corresponded to a  $p_H$  between 3 and 4. The filtrate was kept for nitrogen determination and the precipitate purified as outlined above by dissolving in 0.1*N* sodium hydroxide and reprecipitating by hydrochloric acid, the filtrates from each reprecipitation being analysed for nitrogen and phosphorus. The data thus obtained served for the calculation of the P/N ratios (see Table I) and when this became constant the product was dehydrated by means of alcohol, dried over sulphuric acid *in vacuo* and a portion taken for analysis.

#### *Phosphorised caseinogen.*

In view of the fact that evidence has been obtained by Rimington and Kay [1926] suggesting the existence of an ester linkage in caseinogen between phosphoric acid and some other constituent of the molecule, it became of interest to find out whether by artificial means still further quantities of phosphorus could be induced to combine with the protein and, if this took place, to ascertain if possible the nature of the combination.

A quantity of 18 g. was dissolved in sufficient sodium hydroxide to give a neutral solution and the volume made up to 250 cc. Using the precautions described above, 50 g. of phosphorus oxychloride dissolved in 200 cc. of carbon tetrachloride were then added to the ice-cold solution in such a way that the operation was completed in 9 hours, sodium hydroxide solution being added as required to maintain a faintly alkaline reaction.

The phosphorised product was precipitated by addition of 59.5 cc. of 2*N* hydrochloric acid, filtered off, washed with a litre of distilled water and re-dissolved in the minimum quantity (87 cc.) of 0.2*N* sodium hydroxide. The exact equivalent of hydrochloric acid (0.2*N*) was then added very slowly to the rapidly stirred solution and the process of filtration, washing, etc., repeated until purification was complete as shown by the constancy of the P/N ratio in the filtrate from the precipitated protein. As a rule, five or six reprecipitations were found necessary before this stage was reached.

Table I.

Purification of phosphorised caseinogen. Nitrogen and phosphorus contents of the filtrates from each reprecipitation.

Filtrate	Volume cc.	Total nitrogen mg.	Total phosphorus mg.	P/N
1	300	31.2	--	—
2	242	4.55	5.79	1.27
3	320	6.53	2.83	0.43
4	248	6.35	1.24	0.20
5	266	5.43	1.21	0.22
6	300	3.83	0.60	0.16

The product was finally dehydrated by alcohol, and dried over sulphuric acid *in vacuo*. The yield was 61 %. Upon analysis it gave the following results, from which it will be seen that a further 0.97 % of phosphorus had been introduced into the molecule.

#### Analysis

H <sub>2</sub> O lost at 107° (% of wet weight)	...	...	...	7.72
Total N (Kjeldahl, % of dry weight)	...	...	...	13.53
Total P (Neumann, % of dry weight)	...	...	...	1.77

Ratio P/N = 0.130. A second preparation gave P/N = 0.131.

The substance in all appearances resembles caseinogen but requires nearly twice as much alkali to bring it into solution.

It was shown by Rimington and Kay [1926] that whereas the phosphorus of caseinogen was quite stable to 0.25*N* hydrochloric acid at 37° and to bone phosphatase preparations, the action of 0.25*N* sodium hydroxide at 37° brought about the liberation of the whole of the phosphorus as inorganic sodium phosphate in 24 hours. Phosphorised caseinogen when tested in the same way gave the following results:

Trypsin	...	...	Slow liberation of inorganic phosphorus.
Pepsin	...	...	Very slow liberation of inorganic phosphorus.
Bone phosphatase	...	...	No liberation of inorganic phosphorus.
0.25 <i>N</i> hydrochloric acid	...	...	Very slow liberation of inorganic phosphorus. Slightly slower than with pepsin.
0.25 <i>N</i> sodium hydroxide	...	...	Complete liberation of phosphorus as phosphate in 24 hours.

Phosphorised caseinogen is also coagulated by rennin in the presence of calcium chloride, a fact which seems to show that whatever modification the treatment with phosphorus oxychloride may have produced, no radical structural change can have taken place, such as, for example, destructive hydrolytic cleavage.

*Re-phosphorised caseinogen.*

The name "dephosphorised caseinogen" was given by Rimington and Kay to the phosphorus-free protein obtained by allowing 1 % sodium hydroxide to act upon caseinogen for 24 hours at 37°. The substance so formed resembles caseinogen, but has an appreciably lower nitrogen content. An analysis of dephosphorised caseinogen by the Van Slyke N-distribution method (see Rimington [1927]) has shown that the loss has occurred chiefly in the amide nitrogen, but also to some extent in the arginine fraction, this amino-acid having presumably been partially converted into ornithine and ammonia.

Dephosphorised caseinogen is not coagulated by rennin (one sample did, however, show slight coagulation after many hours), but appears in all other respects to be very similar to caseinogen, except for less ready hydrolysis by proteolytic enzymes, due no doubt to partial racemisation having occurred during the action of the alkali.

4.7 g. of a preparation of dephosphorised caseinogen were phosphorised by means of 13 g. of phosphorus oxychloride in 75 cc. of carbon tetrachloride and yielded a substance, here referred to as "re-phosphorised caseinogen."

*Analysis*

Total N (Kjeldahl)	...	...	...	14.16 %	} P/N = 0.124
Total P (Neumann)	...	...	...	1.75 %	

It was not appreciably acted on by proteolytic enzymes nor by bone phosphatase preparations, but lost all its phosphorus as phosphoric acid in 24 hours when acted upon by 0.25*N* sodium hydroxide at 37°. Towards 0.25*N* hydrochloric acid it displayed the same behaviour as phosphorised caseinogen.

Re-phosphorised caseinogen was found to be coagulated by rennin in the presence of calcium chloride, although less rapidly than is caseinogen under the same conditions. Possibly the property of coagulability by rennin is a feature dependent upon the presence in the molecule of organically bound phosphorus. That the nature of the protein is also of importance is what would have been expected *a priori*. Phosphorised globulin prepared by the phosphorylation of alcohol-denatured serum globulin yields insoluble precipitates at once on the addition of soluble calcium salts and one is led to wonder whether the obscure modification of physical properties brought about by the action of rennin upon caseinogen solutions is not, in reality, a process analogous to the denaturation of albumins and globulins by such agents as alcohol, heat, etc.

*Phosphorised globulin.*

All samples of protein used in this preparation were denaturated by addition to their aqueous solutions of 5 volumes of alcohol and 30 cc. per litre of a salt buffer mixture<sup>1</sup>. The protein was then purified by frequent reprecipitation, brought into solution in dilute sodium hydroxide and phosphorised in the usual way. It was found, however, that during the phosphorylation of globulin there is persistently formed a small quantity of material which is only soluble with difficulty in dilute acids and alkalis. This substance, unless removed by centrifugation, renders the subsequent operations involved in the purification of the phosphorised globulin somewhat difficult. It contains phosphorus in about the same proportion as phosphorised globulin but has not been further investigated.

The dried phosphorised globulin is a fine, white powder, insoluble in water, but dissolved by addition of sodium hydroxide. Its solutions give an immediate precipitate on addition of calcium chloride solution.

*Analysis*

H <sub>2</sub> O lost at 107° (0% wet weight)	...	...	...	10.19
Total N (Kjeldahl, 0% dry weight)	...	...	...	13.85
Total P (Neumann, 0% dry weight)	...	...	...	0.71
Ratio P/N = 0.051				

In order to investigate the stability of the combined phosphorus towards enzyme preparations, etc., solutions of phosphorised globulin were incubated at 37° with the different enzymes at the requisite  $p_H$  and in presence of suitable buffers. Specimens were taken at intervals, and inorganic phosphorus was determined colorimetrically by the method of Bell and Doisy.

The results, with the exception of the action of trypsin, are summarised below:

Bone phosphatase	...	...	No inorganic P formed.
Pepsin	...	...	Very slow liberation of inorganic P, 40 % as PO <sub>4</sub> in 10 days.
Bone phosphatase after pepsin...	...	...	No liberation of inorganic P.
0.25N hydrochloric acid	...	...	Very slow liberation of inorganic P, 35 % as PO <sub>4</sub> in 10 days.
0.25N sodium hydroxide	...	...	Complete liberation of P as PO <sub>4</sub> in 24 hours.

*The action of trypsin on phosphorised globulin.*

It was shown by Rimington and Kay that trypsin very rapidly liberates the whole of the phosphorus from caseinogen in the form of a diffusible, organic compound which is not precipitated by trichloroacetic acid. This substance is very slowly acted upon by trypsin with the formation of inorganic phosphoric acid, but is hydrolysed with extreme rapidity by bone phosphatase preparations, which split off approximately 67 % of the phosphorus as PO<sub>4</sub>. The remaining 33 % may be liberated by means of kidney phosphatase

<sup>1</sup> Made up as follows:

Sol. A. 40 g. potassium sulphate, 202.5 g. sodium sulphate (cryst.), 360 cc. water heated up to effect solution then cooled.

Sol. B. Normal acetic acid.

Sol. C. Normal sodium acetate.

Equal volumes of A, B and C are mixed:  $p_H = 4.7-4.8$ .

preparations. Neither kidney nor bone phosphatase, however, has any appreciable action upon the phosphorus of caseinogen itself.

Phosphorised globulin was investigated in a similar manner. It was found that, in this case also, *trypsin brought about a very rapid liberation of the entire phosphorus in the form of a soluble organic compound*, but only brought about the subsequent hydrolysis to free phosphoric acid at an extremely slow rate. Bone phosphatase preparations, although they have, as just mentioned, no action upon phosphorised globulin, produced, when added to the solutions containing this organic, soluble derivative, *a liberation of the entire phosphorus as phosphoric acid within 10 hours*. This is shown in the accompanying curve, Fig. 1, the curve obtained from caseinogen using the same enzyme preparations being shown for comparison in Fig. 2.

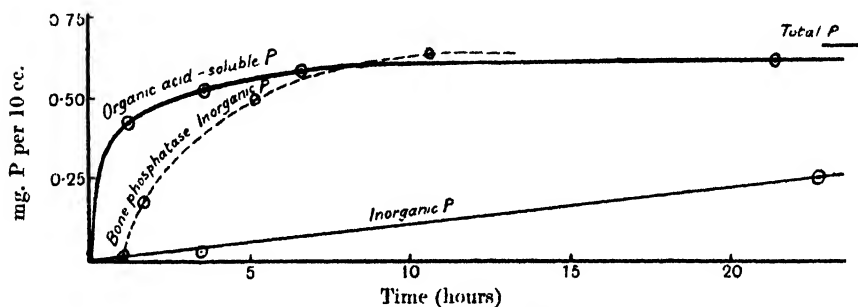


Fig. 1. Action of trypsin and of bone phosphatase on phosphorised globulin.  
 ——— trypsin.      - - - bone phosphatase.

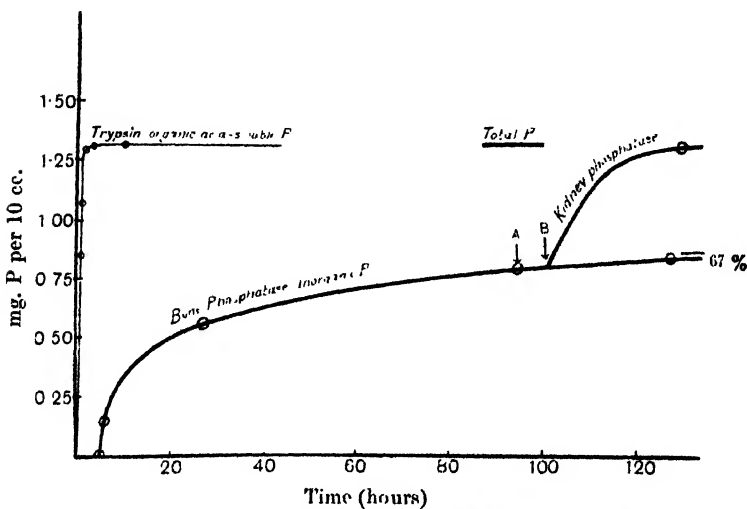


Fig. 2. Action of trypsin, bone and kidney phosphatase on caseinogen.  
 At A a further quantity of bone phosphatase was added.  
 At B kidney phosphatase (rabbit) was added.

It would appear therefore that a similarity exists between the nature of the phosphorus linkage in phosphorised globulin and in caseinogen, with the exception that, in the latter, a portion (1/3rd) of the total phosphorus differs from the remainder in being stable towards bone phosphatase, although hydrolysed by kidney phosphatase preparations.

*The nature of the linkage involved in the phosphorus-containing proteins.*

While as yet no decisive evidence is available, the work already published would render it highly probable that in caseinogen phosphorus is held, in part at least, by means of an ester linkage between phosphoric acid and some constituent of the caseinogen molecule. The existence of a possible NH-P linkage between an amino-group and phosphoric acid seems to have been disproved by the experiments of Rimington and Kay [1926], and evidence is also presented here against the view that such a combination takes place when proteins are phosphorised by phosphorus oxychloride<sup>1</sup>.

Should such a phosphorus-nitrogen linkage exist, it should, on hydrolysis, give rise to a free amino-group titratable by means of formaldehyde according to the Sørensen technique. In order to test this possibility, suitable quantities of the phosphorised proteins were incubated at 37° with 0.25*N* sodium hydroxide for 24 hours under which conditions the whole of the phosphorus is removed as phosphate. The solutions were then aerated for two or three hours at room temperature in order to remove any ammonia [see Rimington and Kay, 1926] and the amino-nitrogen was determined by formaldehyde titration, a sample of the mixture having been titrated immediately after the addition of the sodium hydroxide. In no case was any increase in titratable groups found which was outside the error of the method. This fact in conjunction with the ready hydrolysis by the phosphoric esterase of bone would indicate that the phosphorus has entered the molecule in combination with some hydroxyl group.

Now the number of free hydroxyl groups in proteins is not known, although within the past few years several hydroxy-derivatives of previously known amino-acids have been found to be integral constituents of the protein molecule. Thus Dakin [1918] has estimated the amount of hydroxyglutamic acid in caseinogen as 10 % so that it is probable that the number of free hydroxyl groups is sufficient to account for the quantities of phosphorus which proteins are found capable of taking up. The method might even serve as some guide to the number of free hydroxyl groups present.

The possibility must be borne in mind, however, of the existence in the protein molecule of diketopiperazine rings which, in the "enol" form would be capable of taking up phosphorus just as the hydroxyl group of an amino-

<sup>1</sup> *p*-Chloroanilinephosphinic acid—in which phosphorus is directly linked to nitrogen—was found to be comparatively stable in alkaline solution but was decomposed rapidly with liberation of phosphoric acid by 0.25*N* hydrochloric acid at 37°. Neither trypsin nor phosphatases had any action on this substance.

acid. Whether these cyclic structures do exist, as maintained by Abderhalden and Komm [1924], in natural proteins, and if so in what form, are questions which are as yet undecided, but even supposing an equilibrium to exist between "keto" and "enol" modification, it is quite conceivable that phosphorus oxychloride, by combining with the "enol" form might so alter the equilibrium that eventually all the diketopiperazines pass over into this type. In this connection it is of interest to recall the stability of the diketopiperazine ring to trypsin and to compare it with the resistance offered by the organic, soluble phosphorus compounds of caseinogen and of phosphorised globulin towards this enzyme.

According to Bergmann [1924], a certain lability characterises the derivatives of the amino-acid serine, in which a benzoyl group has been introduced in place of hydrogen. Bergmann found that so slight a change as that of the medium from acid to alkaline reaction was sufficient to cause migration of the benzoyl group from union with the hydroxyl to the nitrogen atom.

As a result of his investigations, he is led to the view that such interchanges may take place in the protein molecule, thus furnishing a possible explanation of the extreme lability characteristic of protein material. One cannot discount the possibility that a phosphoryl group might behave in a manner at any rate analogous to the benzoyl group, although, up to the present, no experimental fact has been encountered supporting such an opinion.

#### SUMMARY.

1. Proteins may be phosphorised by means of phosphorus oxychloride in a manner analogous to the Schotten-Baumann reaction.

2. Caseinogen, serum globulin and dephosphorised caseinogen have been treated in this way. Phosphorised caseinogen contains 1.77 % P, corresponding to a ratio  $P/N = 0.130$ . "Re-phosphorised caseinogen," the product obtained from dephosphorised caseinogen, contains 1.75 % P and has a ratio  $P/N = 0.124$ . Both substances are coagulable by rennin.

3. Phosphorised globulin contains 0.71 % P with  $P/N = 0.051$ . It is precipitated by soluble calcium salts.

4. The phosphorised proteins lose phosphorus slowly as phosphoric acid under the action of pepsin or 0.25 *N* hydrochloric acid, but 0.25 *N* sodium hydroxide at 37° liberates the whole of the phosphorus as phosphoric acid in 24 hours. Bone phosphatase liberates *no* inorganic phosphorus.

5. Trypsin brings about a rapid separation of the whole of the phosphorus of phosphorised globulin in the form of an organic, acid-soluble substance. It only liberates phosphoric acid from the compound with extreme slowness.

6. Bone phosphatase liberates the whole of the phosphorus from this organic substance as phosphoric acid within a few hours. In this respect, phosphorised globulin exhibits a similarity to caseinogen.

7. Phosphorus appears to enter the protein by means of an ester linkage between phosphoric acid and some hydroxyl group.

In conclusion I wish to thank Professor S. P. L. Sørensen of the Carlsberg Laboratorium, Copenhagen, for the great kindness and hospitality shown to me when working in his laboratory where the greater part of this work was done and for much helpful criticism and advice received from him. My thanks are also due to Sir F. G. Hopkins for the interest he has taken in the latter part of the work.

I am indebted to the Department of Scientific and Industrial Research for an allowance towards travelling expenses.

## REFERENCES.

- Abderhalden and Komm (1924). *Z. physiol. Chem.* **139**, 181; and later papers by Abderhalden.  
Bechhold (1901). *Z. physiol. Chem.* **34**, 122.  
Bergmann (1924). *Naturwiss.* **12**, 1158.  
Crowther and Raistrick (1916). *Biochem. J.* **10**, 434.  
Dakin (1918). *Biochem. J.* **12**, 290.  
Liebermann (1888). *Ber. deutsch. chem. Ges.* **21**, 598.  
Neuberg and Oertel (1914). *Biochem. Z.* **60**, 491.  
Neuberg and Pollak (1910). *Biochem. Z.* **26**, 529.  
Pohl (1889). *Z. physiol. Chem.* **13**, 292.  
Rimington (1927). *Biochem. J.* **21**, 204.  
Rimington and Kay (1926). *Biochem. J.* **20**, 777.  
Woodman (1921). *Biochem. J.* **15**, 187.



## XL. THE DIETARY VALUE OF POTATO PROTEIN.

BY GLADYS ANNIE HARTWELL.

*From the Physiological Laboratory, Household and Social Science Department,  
King's College for Women, Kensington, London, W. 8.*

*(Received January 14th, 1927.)*

In a previous paper [Hartwell, 1925] it was shown that rats fed on a diet of potato, butter and salt mixture grew slowly and exhibited loss of fur. It was suggested that the use of new potatoes instead of old ones might lead to better results, and therefore further experiments on the same lines were started. Some years previously McCollum, Simmonds and Parsons [1918] fed young rats on potato supplemented in various ways. The potato used by these workers was dried, and ground up, so that the experiments are not strictly comparable with those described in this paper, where the object was to feed the potato in a form in which it is taken by man. McCollum and his co-workers found that the potato was poor in calcium, sodium and chlorine; therefore to guard against a shortage of any mineral constituents, salt mixture [Hartwell, 1922] was used in the diet described in this paper. Potatoes appear to contain quite large quantities of vitamin B [Steenbock and Gross, 1919] and Osborne and Mendel [1920, 1] find that there is no appreciable difference between old and new potatoes in respect of this factor. With regard to vitamin A, there appears to be some difference of opinion. Steenbock and Gross [1919] found that one lot of potatoes contained sufficient fat-soluble vitamin to support growth in young rats, while another sample contained none. This discrepancy in results must surely be due to some difference in technique not realised in this earlier work. Osborne and Mendel [1920, 2] find that the potato as a whole cannot be entirely devoid of fat-soluble vitamins. It seemed, therefore, safer to give butter fat in the diet and thus to guard against any possible deficiency. Incidentally the fat was useful for increasing the calorie value of the food.

### *Experimental.*

*Diet.* The diet consisted of potato, butter, salt mixture and water. The potatoes were boiled in their skins and then peeled and mashed. The proportions used were 100 g. boiled potato, 1 g. salt mixture, 5 g. butter and 25 cc. of water to make a stiff paste. Food was given *ad lib.* and distilled water was provided in the usual water bottles.

*Exp. 1. Effect of the potato diet during growth.* 12 young rats, 6♀ + 6♂, were given the potato diet from weaning (3 weeks and 2 days). The experiment lasted just over 3 months, from April 14 to July 21, 1924. Old potatoes were used. The average ♂ curve is given in a previous paper [Hartwell, 1925].

*Exp. 2. Effect of the potato diet during growth, gestation and lactation.* This experiment was the same as Exp. 1, except that new potatoes were used instead of old ones and the feeding was continued for a longer period (August 18, 1924–May 21, 1925).

*Exp. 3. Effect of the potato diet during lactation only.* Six stock does were fed on a mixed diet of kitchen scraps and bread and milk until litters were born, after which they were given the potato diet.

*Exp. 4. Effect of the potato diet supplemented with bread, during lactation only.* Six stock does similar to those used in Exp. 3 were given a diet consisting of 15 g. potato diet, 15 g. bread and 21 cc. water mixed well together. The feeding was started as soon as possible after the birth of the litter.

*Method.* In Exps. 1 and 2 the method used was that described in a former paper [Hartwell, 1926]. For Exps. 3 and 4 the technique has been fully described in earlier papers on mammary secretion.

## RESULTS AND DISCUSSION.

*Growth.* In Exps. 1 and 2 most of the young rats grew, but very slowly compared with control animals. Two of the males showed maintenance of weight, but hardly any growth. Their condition was poor, and their fur came out badly [Hartwell, 1925]. It eventually grew again, but the coats were always thin. Rats fed on new potatoes appeared no better than those fed on old ones. In Exp. 2 the animals had an old look at a comparatively young age. That this frequently occurs when rats are fed on a poor diet has been shown by McCollum, Simmonds and Parsons [1918]. The young animals were particularly susceptible to cold, and, as shown in Fig. 1, several of them died after a short period on the potato diet. *Post mortem* the lungs were found to be badly affected in all cases. McCollum, Simmonds and Parsons [1918] fed rats on diets consisting largely of potato, and state that "growth took place slowly or not at all, individual animals shewing somewhat different capacity to make use of the diet." The results described here are quite in agreement with this statement. These observers gave 7-8 % of potato protein in the food (using dried potato), and the diet used by me could not have contained more than 7.6 % of protein. A possible explanation of the failure of growth in some animals is the low protein content, which may be borderline, and hence some of the rats were unable to adjust their metabolism to this low level of intake. That the rats were in fairly good health is shown by the rapid growth and improvement in condition produced by changing the diet. In Exp. 1 the food was changed to a mixture of 15 g. bread, 6 g. food casein [Hartwell, 1922], 0.4 g. salt mixture, 1.5 g. butter and 45 cc. of 3 % marmite.

After 10 days the animals were in good condition; the fur was thick and glossy and they had grown much more rapidly.

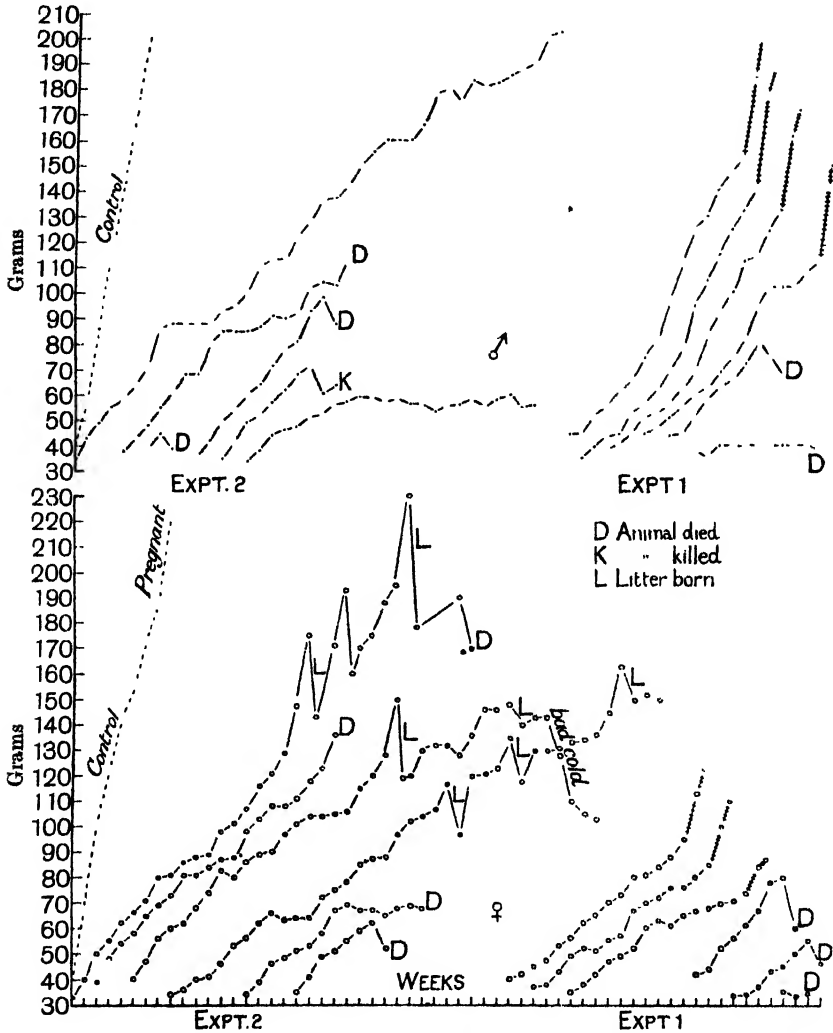


Fig. 1. Growth curves of young rats fed on a diet of potato, butter, salt mixture and water.

... Diet changed to bread, food casein, salt mixture, butter and marmite.

*Maintenance.* No experiments were made to test the efficiency of potato protein as regards maintenance, but it seems reasonable to suppose that a diet which provides for growth (and reproduction), at any rate in some animals, is adequate for maintenance. Rose and Cooper [1917] found that a woman was able to maintain nitrogenous equilibrium for 7 days on a diet of potato, sugar, and agar-agar; they conclude, therefore, that the nitrogenous compounds of the potato possess a high nutritive efficiency.

Hindhede [1913] considers that "vegetable foodstuffs, and potatoes in particular, are considerably better digested than animal." He calculates (from the experiments of Constantinidi on man) that of 3000 calories in potatoes and fat, only 2 % go to waste in the faeces, while in meat and fat, or milk, the loss is 5 %.

*Reproduction.* No young were born until the does were about 5½ months old, while stock animals produce and rear their young as early as 3 months. An explanation of this probably lies in the fact that the rats on the potato diet were very small for their age and of poor weight. The breeding results were as follows:

- Rat 1<sup>1</sup>. Produced 3 litters.
  - 1. 3 + 1 dead—all eaten 11th day.
  - 2. 1 + 7 dead—dead 6th day.
  - 3. 7 + 4 dead—3 reared—average weight at 21 days 36.3 g.
- Rat 2<sup>1</sup>. Produced 3 litters.
  - 1. 1 + 3 dead—dead 2nd day.
  - 2. Born dead.
  - 3. Born dead.
- Rat 3. Died before any young born.
- Rat 4. Produced 2 litters.
  - 1. 3—eaten 3rd day.
  - 2. Eaten 2nd day.
- Rat 5. Died before any young born.
- Rat 6. Died at birth of 1st litter.

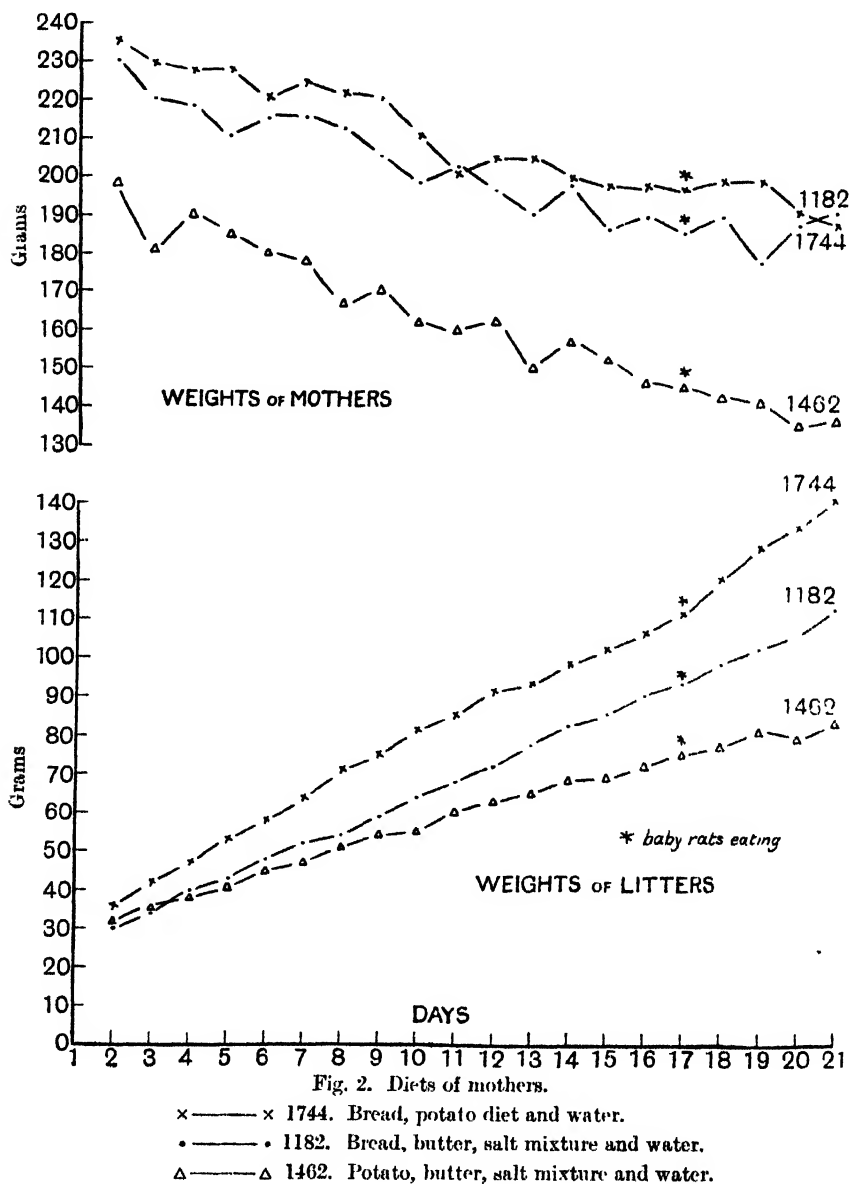
The rats fed exclusively on the potato diet were not sterile, but the food must be regarded as very unsatisfactory for gestation, because so many of the young were born dead. The noticeable failure to rear the young (even when a good diet, such as bread and milk, was given to the mother rat after their birth) suggests that those which were alive were weakly. All the young were of low weight at birth. The low percentage of protein in the diet may account for these poor results.

*Lactation.* Exp. 2 gave no results in this respect; only one litter was reared and the mother had been given bread and milk.

Exp. 3 showed that it was possible for the rat to rear her young when she was fed on the potato diet during lactation. Each rat was allowed to keep six of her babies, but only one animal succeeded in bringing up the whole litter. The growth curve of these is shown in Fig. 2. The other rats lost two or three of their young at different stages of lactation. From Fig. 2 it is obvious that the mother rat lost weight during lactation. This loss of weight occurred in all cases. The young grew slowly, and were below normal weight at weaning; their fur was thin and poor. The general condition of the young was similar to that obtained when the mother rat was fed on a diet of bread, butter and salt mixture, but with the bread diet the weight curve of the young was slightly better, though the mother rat lost weight as badly. (1182 in Fig. 2 is put in for comparison.)

<sup>1</sup> Bread and milk diet given after the birth of the litter.

In Exp. 4 bread was added to the potato diet, and, as seen from the figure, the growth of the young rats was improved. Their general condition also was better, but they were still far below average weight, being only 23-24 g. at weaning instead of approximately 40 g. The supplementing of the potato and bread proteins is in agreement with the work of McCollum, Simmonds and Parsons [1921].



These experiments show that potato as the sole source of protein in the diet is not adequate to meet the needs of the rat. Is it the *quality* or the

*quantity* of the protein which is at fault? McCollum, Simmonds and Parsons [1918] consider that it is the *quality* which is the limiting factor. These workers used other diets "similar in every respect except that a portion of the carbohydrate was replaced by the purified protein casein." This modification led to rapid growth of the animals, but the author does not consider this a proof of any inadequacy in *quality*, because from the statement quoted above it is clear that a greater *quantity* of protein was used. In the experiments described in this paper, the percentage of protein in the food was only about 7.6, much too low to expect good growth. Therefore the fact that growth and even reproduction took place seems to suggest that the potato protein is of good quality and it is the insufficient amount which caused the poor results. In support of this hypothesis there is the work of Rose and Cooper [1917] and the observations of Hindhede [1913] previously quoted. Onslow [1920] states that potatoes contain a globulin, tuberin; such proteins are regarded as being of good quality. Further, tyrosine, lysine and leucine are said to be found in the tubers [Onslow, 1920]. According to Rose and Cooper [1917] only 63 % of the potato nitrogen is in the form of protein, but the animal organism is capable of using amino-acids, and tyrosine is an essential member of this group. A watery, protein-free extract of potato gives a well-marked Millon's reaction.

The calorie value of the potato diet was equivalent to that eaten by other rats giving normal growth curves.

#### SUMMARY.

1. Potato protein provides for growth in some rats but the rate is very slow. Other rats fail to grow on the diet.
2. Reproduction takes place on a diet of potato, butter, salt mixture, and water, but many of the young are born dead, and hardly any of them are reared.
3. The rat can rear her young on the above diet (if she is given better food during gestation), but the babies are undersized and have poor coats; the mother rat loses weight during lactation.
4. The addition of bread to the potato diet during lactation results in improved growth and condition of the young rats, but the mother still loses weight.
5. It is suggested that the *quantity* rather than the *quality* of the potato protein is responsible for the slow growth.

The expenses of this research were defrayed by a grant from the Medical Research Council.

## REFERENCES.

- Hartwell (1922). *Biochem. J.* **16**, 78.  
—— (1925). *Biochem. J.* **19**, 75.  
—— (1926). *Biochem. J.* **20**, 751.  
Hindhede (1913). Protein and Nutrition.  
McCollum, Simmonds and Parsons (1918). *J. Biol. Chem.* **36**, 197.  
—— ——— (1921). *J. Biol. Chem.* **47**, 139.  
Onslow (1920). Plant Biochemistry.  
Osborne and Mendel (1920, 1). *J. Biol. Chem.* **41**, 451.  
—— ——— (1920, 2). *J. Biol. Chem.* **41**, 549.  
Rose and Cooper (1917). *J. Biol. Chem.* **30**, 201.  
Steenbock and Gross (1919). *J. Biol. Chem.* **40**, 501.

## XLI. THE PHOSPHORUS NUCLEUS OF CASEINOGEN.

By S. POSTERNAK.

*From the Private Laboratory of the Author, Chêne-Bougeries, Geneva.*

*(Received January 21st, 1927.)*

In an interesting paper by Rimington and Kay [1926] entitled "The liberation of phosphorus from caseinogen by enzymes and other agents." I noticed the following sentence:

"We could find no account in the literature of any serious attempt to isolate the organic phosphorus compound or compounds produced during tryptic hydrolysis of caseinogen."

I think it my duty to call the attention of the authors to the fact that as early as 1923 the Society of Chemical Industry in Basle patented a process of mine for preparing the phosphorus nucleus of caseinogen by means of a short tryptic digestion (2-3 days) [1923, 1925].

As Rimington and Kay will easily realise, if they study the publication alluded to, I was able to isolate from the tryptic digestion liquor of caseinogen, with the aid of its lead salt, a phosphorus compound containing 5.86 % P and 11.90 % N. *i.e.* 4 atoms P for 18 atoms N.

The compound obtained by Rimington and Kay contains only 3.8-4.00 % P.

Later on, I succeeded in preparing more degraded polypeptides and determined their constitution. According to these researches the phosphorus nucleus of caseinogen is composed of four serine-phosphoric acids.

More details concerning the properties and physiological meaning of these phosphopolypeptides are to be found in my other publications [1926, 1927].

### REFERENCES.

- Posternak (1926). *Compt. Rend. Soc. Physiol. Genève*, **43**, 16.  
— (1927). *Compt. Rend. Acad. Sci.* **184**, 306.  
Rimington and Kay (1926). *Biochem. J.* **20**, 777.  
Society of Chemical Industry, Basle (1923). Swiss Patent No. 104336,  
25th Jan. 1923.  
— — (1925). *Chem. Zentr.* **1**, 1371.



## XLII. CONTRIBUTIONS TO THE STUDY OF LIGNIN.

### PART I. METALIGNIN, A NEW TYPE OF ALKALI LIGNIN.

BY CHARLES DORÉE AND EUSTACE CECIL BARTON-WRIGHT.

*From the Chemistry Department, Chelsea Polytechnic, S.W.*

*(Received January 29th, 1927.)*

ALTHOUGH the soda process for the resolution of lignified material into cellulose, and soluble derivatives of lignin, is an important industrial process, very little evidence has been recorded as to the nature and composition of the lignin so obtained, and of the relation existing between this form of lignin and the lignosulphonic acids obtained in the bisulphite process.

Powell and Whittaker [1924, 1925], who investigated the lignins obtained from flax and a number of woods, including pine, spruce, ash, birch and poplar, employed a solution of caustic soda of 8-12 % concentration for six to ten hours at 140-160°. They considered that this process does not involve internal changes in the lignin molecule, and that the lignin so obtained is "probably closely related to lignin as it exists in the lignocellulose complex." The results of a large number of comparative experiments showed that the lignin isolated from flax, and from the various woods under the above conditions, is of constant composition, viz.  $C_{45}H_{46}O_{16}$ , and the authors concluded that plant lignin is derived from a basic substance which they term "lignol,"  $C_{41}H_{40}O_{16}$ . Lignol contains nine hydroxyl groups and the authors assume that the lignins present in the various woody tissues differ only in the extent to which the hydroxyl groupings of the basic lignol are methylated. The lignin  $C_{45}H_{46}O_{16}$  contains four methoxyl and five hydroxyl groupings and the product obtained from spruce-wood showed on analysis the composition  $C = 64.0$ ,  $H = 5.5$  %.

There is no doubt that the working conditions, the time and temperature and the concentration of alkali used influence the resulting product. Under the prolonged action of alkali at high temperatures internal changes, and probably oxidation of the lignin complex, take place [Fuchs, 1926]. The data obtained by Beckmann, Liesche and Lehmann [1923] illustrate this variability in composition.

Beckmann, Liesche and Lehmann [1921] have employed perhaps the most restrained conditions in isolating lignin fractions from rye-straw; namely, 2 % sodium hydroxide in aqueous-alcoholic solution acting in the cold for

48 hours. The alcohol prevented the solution of hemicelluloses, pentosans and other non-lignified substances, which are soluble in, and removed by, aqueous alkalis.

Their product was a yellow powder which darkened and aggregated at 50°. Its molecular weight was of the order of 800 and its composition approximated to the formula,  $C_{40}H_{44}O_{15}$ , this unit containing 4 hydroxyl and 4 methoxyl groupings.

The two investigations cited illustrate extreme variations in working conditions. Other products obtained from commercial alkaline black liquors have been described [see Hägglund, 1924; Holmberg, 1921].

Apart from oxidation and definite internal change brought about by the prolonged action of caustic alkali, removal of one or more methoxyl groupings of the original lignin is probable, resulting in the isolation either of a single derivative, or of a mixture of partly demethylated derivatives. In order to obtain a lignin as near as possible to its original condition, it is desirable, therefore, to reduce the severity of the alkaline attack and shorten the time as far as consistent with satisfactory resolution.

In the course of experiments having for their object the quantitative separation of lignin from lignified tissues, Mehta [1925] found that sodium hydroxide solution in a concentration of 4 % gave the best result, and that with this strength of solution at a pressure of 10 atmospheres the resolution of the wood was complete in one hour. The lignin is stated to be isolated quantitatively on acidifying the solution.

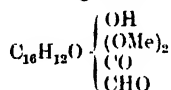
The method was used by this author for the estimation of lignin in a large number of plant products—the values obtained being very much lower than those generally found by the hydrochloric acid method of Willstätter and Zechmeister [1913]. The lignin obtained from different species of wood was found to be a brown powder with the odour of vanillin, insoluble in water, but soluble in dilute alkalis, ammonia, alcohol, acetone and glacial acetic acid. It differed from all lignins previously described in that it had a fairly definite melting point (170°). The acid value was found to be 477 and the iodine value 139.7.

The method appeared to us likely to yield a lignin with the minimum of chemical change, while the marked solubility in a range of organic solvents and the evidence of a definite melting point seemed to indicate a lignin of relatively simple composition, in agreement with our own results on jute fibre. By arrangement with Dr D. N. Nanji, therefore, we have carried out an investigation of the substance, the results of which are given below.

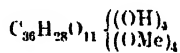
It has been overlooked by most previous workers that caustic alkali removes from the wood not only lignin, but also resins, fat-waxes and hemicelluloses which it may be impossible to remove subsequently from the lignin and this may very well account for the variable compositions reported by different workers. The wood used throughout this investigation was spruce, which was thoroughly extracted with benzene, alcohol and water and then digested

with 5 % sodium hydroxide solution in the cold to remove hemicellulose. The lignin isolated by us was a brown powder, soluble in most organic solvents with the exception of ether, benzene and light petroleum. Its melting point was 186°. It was soluble in sodium carbonate, but not in ammonium carbonate solutions. It had the composition  $C_{20}H_{20}O_6$ , and determinations of the molecular weight gave the somewhat surprising result that this formula represented the molecular unit<sup>1</sup>. We have thus, as anticipated, a compound of relatively simple composition; almost all lignins previously isolated having been assigned formulae with approximately double this number of carbon atoms. As the product is possibly a depolymerised form of the lignin as it exists in the wood and as it exhibits definite properties by which it can be identified, we propose to refer to it as *metalignin*.

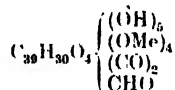
Metalignin,  $C_{20}H_{20}O_6$ , has one free hydroxyl group, two methoxyl groups and, according to the composition of the oxime it contains, two carbonyl groups, one of which may be ketonic and the other aldehydic. The iodine value of 139.7, previously recorded, corresponds to two unsaturated linkages in the molecule (found:  $C_{20}H_{20}O_6$  of molecular weight 356 absorbs iodine 498.4,  $2I_2$  requires 508). It is interesting to note in this connection, that Cross and Bevan [1882] isolated a small alcohol-soluble fraction from chlorinated jute which had the composition  $C_{19}H_{18}O_9Cl_4$  with one free hydroxyl grouping. These results together with those recorded for the alkali lignins are summed up in the following schematic formulae:



I. Metalignin  
 $C_{20}H_{20}O_6$



II. Beckmann, Liesche and  
Lehmann: alkali lignin  
from rye-straw  $C_{46}H_{31}O_{15}$



III. Powell and Whittaker:  
lignin  $C_{45}H_{46}O_{16}$

### *Preparation of metalignin.*

Spruce-wood was employed throughout the present work as its constants are well known and the majority of the lignin derivatives previously described in the form of sulphonic acids were prepared from this wood. An analysis of the wood employed will be found on p. 299, from which it will be seen that it contains 5.8 % of extractives and 3.8 % of hemicelluloses. The purification of the wood followed generally on the lines recommended by Friedrich and Diwald [1925].

The wood, in the form of fine sawdust, was well extracted with benzene (12 hours), alcohol (12 hours) and water (12 hours) to remove resins and glucosides, and then digested with four changes of a 5 % solution of sodium hydroxide for 36 hours to remove hemicelluloses (for quantitative results see p. 299). The metalignin itself was isolated by heating the purified sawdust in an autoclave with a 4 % sodium hydroxide solution at 8 atmospheres

<sup>1</sup> It is interesting to note that Holmberg [1921], by the extraction of sulphite liquor with ether, has isolated a crystalline substance, m.p. 250°, of the same formula  $C_{20}H_{20}O_6$ , which is probably the lactone of diguaiacyltetramethylenecarbinolcarboxylic acid.

pressure for one hour<sup>1</sup>. The dark solution of sodium metalignate was poured off and the metalignin precipitated by the addition of hydrochloric acid, washed by decantation, dissolved in 95 % alcohol and the alcoholic solution evaporated to dryness. The metalignin thus obtained was a lustrous black solid.

It was purified by solution in glacial acetic acid and precipitation with water; the precipitated product was washed with water and dried at 60°. After the first precipitation the original value for the melting point, 170°, was raised to 180°, whilst after the second and third treatment the melting point remained constant at 185–186°.

#### *Properties of metalignin.*

The purified metalignin was a light brown, odourless solid, easily soluble in pyridine, alcohol, acetone, glacial acetic acid, ethyl acetate, naphthalene, trichloroacetic acid, saturated calcium thiocyanate solution and slightly soluble in chloroform. It was insoluble in ether, benzene, ligroin, light petroleum, as well as in sodium bisulphite solution, sulphurous acid and ammonium oxalate solution. It dissolved readily in sodium hydroxide, ammonium hydroxide and sodium carbonate solutions and was precipitated from these on the addition of acids. It was insoluble in ammonium carbonate solution. The calcium and barium salts were precipitated on the addition of the soluble salts of these elements to a solution of metalignin in ammonia. In its solubility in glacial acetic acid and acetone, it differed from the product obtained by Powell and Whittaker. The dry substance contained 0.13 % ash. Analyses are calculated on ash-free material.

Found: C = 66.8, H = 5.6; C = 67.0, H = 5.6;  $C_{20}H_{20}O_6$  requires C = 67.3, H = 5.6.

The formula  $C_{20}H_{20}O_6$  for metalignin is the same as that predicted by Klason for  $\alpha$ -lignin (see below). Determinations of the molecular weight by the cryoscopic method in glacial acetic acid gave the average value 341.

0.4410 g. in 31.3 g. glacial acetic acid  $\Delta = 0.164^\circ$  M.W. = 336

0.8770                   "                   "                   = 0.314°                   "                   = 341

1.3186                   "                   "                   = 0.473°                   "                   = 347

The molecular weight was also determined in naphthalene and gave the average value 342.

The molecular formula is therefore equal to  $C_{20}H_{20}O_6$ . Two methoxyl groups were found to be present.

Found: OMe = 18.6 % and 17.4 %;  $C_{18}H_{14}O_4(OCH_3)_2$  requires OMe = 17.4 %.

*Monobenzoyl-metalignin*  $C_{20}H_{18}O_6(CO.C_6H_5)$ . Metalignin reacted with benzoyl chloride in alkaline solution with great ease.

The monobenzoyl-metalignin was a pale yellow, almost white solid, m.p. 184°. It was very soluble in pyridine, less so in alcohol and insoluble

<sup>1</sup> A 10 % solution of sodium hydroxide at 4 atmospheres pressure for 1 hour also gave good results.

in ether, benzene, ligroin and light petroleum. The crude benzoyl derivative was dissolved in pyridine and precipitated with 95 % alcohol. After three such treatments the melting point became constant at 184°.

Found: C = 70.1 %, H = 5.1 %, OMe = 13.8 %;  $C_{27}H_{24}O_7$  requires C = 70.4 %, H = 5.2 %, OMe = 13.4 %.

*Monoacetyl-metalignin*  $C_{20}H_{18}O_6$  (CO.CH<sub>3</sub>). The acetyl derivative was prepared according to the description given by Powell and Whittaker [1924]. 5 g. metalignin and 20 g. acetyl chloride were mixed and a few drops of concentrated sulphuric acid added. The metalignin rapidly went into solution with evolution of a considerable amount of heat, the mixture being heated for one hour at 80°. The acetyl derivative was precipitated by pouring the hot solution into ice-water with vigorous stirring. The acetyl-metalignin was very soluble in pyridine, alcohol and acetone, insoluble in ether, benzene, ligroin and light petroleum. It was purified by dissolving in acetone and precipitating with brine, washing with water and drying at 60°. Unlike the acetyl derivative described by Powell and Whittaker it was quite stable.

Found: C = 66.0 %, H = 5.5 %;  $C_{22}H_{22}O_7$  requires C = 66.3 %, H = 5.5 %.

*Methyl-metalignin*  $C_{18}H_{13}O_3(OCH_3)_3$ . 5 g. metalignin were dissolved in 12 cc. of 10 % caustic soda solution and dimethyl sulphate was added drop by drop with constant shaking. The methylated derivative was slowly precipitated. Purification was effected by dissolving the crude product in pyridine and precipitating with 45 % alcohol. The methylated derivative was a pale, almost canary yellow compound, very soluble in pyridine, acetone and alcohol, insoluble in ether, benzene and light petroleum.

Found: OMe = 25.4 %;  $C_{18}H_{13}O_3(OCH_3)_3$  requires OMe = 25.1 %.

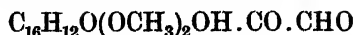
*Metalignin dioxime*  $C_{20}H_{22}O_6N_2$ . The metalignin contains active carbonyl groupings, which was shown by the fact that it reduced Fehling's solution and ammoniacal silver nitrate solution and by the preparation of the dioxime. This compound was prepared by the method described by Brady and Dunn [1913]. 3 g. metalignin were mixed with 3 g. hydroxylamine hydrochloride and an 8 % solution of sodium hydroxide was added gradually to the mixture. The solution was filtered and the oxime precipitated by the addition of ammonium chloride solution.

The oxime was a pale brown solid, soluble in pyridine and acetone, but only slightly soluble in alcohol. It was insoluble in ether, light petroleum and benzene. It was purified from alcohol solution.

Found: N = 6.7 % and 6.9 %;  $C_{20}H_{22}O_6N_2$  requires N = 7.2 %.

*Metalignin-phenylhydrazone*. The preparation of a phenylhydrazine derivative proved unsatisfactory. The metalignin did not react with the phenylhydrazine at all readily, and it seemed doubtful if the compound finally obtained was the true hydrazone. With the exception, perhaps, of the compound obtained by Dorée and Hall [1924] from their lignosulphonic acid, the phenylhydrazones obtained from the various lignins do not stand in any definite relationship to the parent substance.

From the results recorded above, the formula



is given to metalignin. It will be seen that all the oxygen atoms, except one, are accounted for, leaving a nucleus of 16 carbon atoms.

It was hoped that on oxidation some definitely recognisable cleavage product would result. This was not the case.

*Oxidation of metalignin.*

(a) 5 g. metalignin were heated with 100 cc. of 5 % nitric acid on the water-bath for six hours, when an insoluble orange-red compound slowly formed, which proved to be soluble in all the solvents for metalignin itself. From its general reactions it appeared to be similar to the compound  $\text{C}_{25}\text{H}_{27}\text{N}_2\text{O}_{17}\cdot\text{OCH}_3$ , isolated by Dorée and Hall [1924] by the oxidation of lignosulphonic acid with 5 % nitric acid. It gave ammonia on warming with sodium hydroxide and could be reduced with zinc dust and acetic acid to a brown substance. It was more acidic in character than metalignin, dissolving readily in alkalis and ammonium carbonate, from which solutions it could be precipitated on the addition of acid.

(b) 6 g. metalignin oxidised for 4 hours with 10 % nitric acid gave 3 g. oxalic acid. Dorée and Hall also discovered that when lignosulphonic acid was oxidised with strong nitric acid, oxalic acid was obtained in considerable yield.

(c) Metalignin was oxidised with sulphuric acid in the presence of mercuric sulphate at 165°. By this method a minute amount of the mercury salt of an acid was obtained, which was very soluble in ether, alcohol and water, slightly soluble in benzene and insoluble in light petroleum. It was, however, obtained in too small a yield to be identified.

*The relation between the lignins isolated by different methods  
and the original lignin of the plant.*

In Table I are collected data relating to some of the better defined types of lignin that have been described. As these vary in the number of methoxyl groupings they contain and some are sulphonic acids and others acetyl derivatives, the empirical formula for the basic "lignol" has been calculated in each case for purposes of comparison. This "lignol" represents the hydroxylated compound, sulphonic, acetyl and methyl groupings being eliminated. A certain general similarity will be observed among all these lignols. The number of carbon atoms approximates to 36 and the number of carbon and hydrogen atoms present is approximately equal. The number of oxygen atoms varies and in this connection we have noticed that isolated lignin has a remarkable capacity for fixing oxygen (under the action, for example, of nitric acid or hydrogen peroxide) which may account for this irregularity.

The fact that, using a particular method of isolation, e.g. that of No. 7, identical products are obtained from different woods, flax, etc., seems fairly

strong evidence for the assumption that the same lignin substance is present throughout the plant world. According to Friedrich and Diwald [1925] it would appear that in the wood tissue it exists as a methylated derivative. By the action of 17 % hydrochloric acid in the cold they claim to have isolated a lignin without hydrolysis of hydroxyl groupings or subsequent condensation changes which take place under the action of the 40 % hydrochloric acid used by Willstätter.

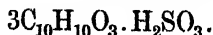
Table I.

Method of isolation*	Author	Empirical formula	No. of OH groups	No. of OMe groups	Corresponding "basic lignol"	Lignol	
						% C	% H
1. 17 % HCl cold	Friedrich and Diwald [1925]	$C_{39}H_{48}O_{14}$	1	5	$C_{34}H_{38}O_{14}$	60.8	5.6
2. 3 vol. HCl (d. 1.18) and 1 vol. $H_3PO_4$ (d. 1.7)	Urban [1926]	$C_{43}H_{48}O_{16}$	5	4	$C_{39}H_{40}O_{16}$	61.2	5.2
3. $H_2SO_3$ aq. at $110^\circ$	Dorée and Hall [1924]	$C_{26}H_{30}O_{12}S$	3 or 4	2	$C_{24}H_{24}O_9$	63.2	5.26
4. Ditto	Klason [1925]	$C_{30}H_{32}O_{12}S$	3 (?)	2 (?)	$C_{28}H_{28}O_9$	66.1	5.1
5. Calcium bisulphite liquor—computed [1920]	Klason $\alpha$ -lignin	$C_{22}H_{22}O_7$	1	2	$C_{16}H_{16}O_6$	65.85	4.84
6. 4 % NaOH at 10 atmos. pressure 1 hour	Dorée and Wright Metalignin	$C_{20}H_{20}O_6$	1	2	$C_{18}H_{18}O_6$	65.85	4.84
7. 8-12 % NaOH at $140-160^\circ$ for 6-10 hours	Powell and Whittaker [1925]	$C_{45}H_{48}O_{18}$	5	4	$C_{41}H_{46}O_{16}$	62.4	5.07
8. 2 % alcoholic NaOH in cold on rye-straw	Beckmann, Liesche and Lehmann [1921]	$C_{40}H_{44}O_{16}$	4	4	$C_{36}H_{36}O_{16}$	61.01	5.08

\* Spruce-wood or allied soft wood unless otherwise stated [Fuchs, 1926, p. 281].

As will be seen (No. 1), this lignin contains 5 methoxy-groupings, of which two are readily removed on saponification and are believed to be ester methoxyl, whilst the remaining three are ether methoxyl.

Metalignin (No. 6) is characterised by melting point and solubility range and it differs from the others in possessing the molecular formula corresponding to a  $C_{20}$  unit, the others approximating to a  $C_{40}$  unit complex. It is interesting to note that Klason had previously deduced that an  $\alpha$ -lignin of the same formula constituted (in the form of its sulphonic acid) one component of the lignin isolated in the calcium bisulphite process. Later [1925], in order to explain the existence of the acid  $C_{26}H_{30}O_{12}S$  (No. 3) which is obtained by the action of sulphurous acid solution at  $110^\circ$ , Klason has doubled the formula for  $\alpha$ -lignin and considers that the acid  $C_{26}$  is a mixture of a mono- and a dibasic sulphonic acid  $2C_{20}H_{20}O_6 \cdot 2H_2SO_3$  and  $2C_{20}H_{20}O_6 \cdot H_2SO_3$  respectively, while in a later communication he states that the simplest acid obtainable by the action of sulphurous acid solution on wood has the composition

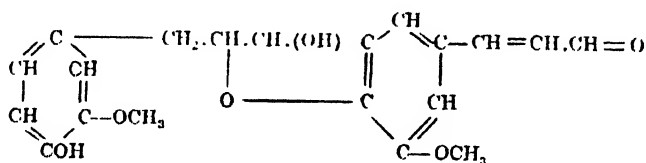


There is thus a connection between the product obtained from spruce by sulphurous acid, and by the action of dilute alkali for a minimum time. It

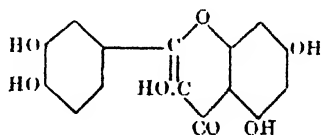
may be that metalignin,  $C_{20}$ , is the unit upon which the natural lignins are based and that, whilst the usual type isolated is of the order  $C_{40}$ , it may exist in the plant in an even more polymerised form. In this way its resistance to oxidation in the living tissue and its susceptibility when isolated may be explained.

With regard to the constitution of metalignin, we do not assume the presence of the acidic (carboxyl) grouping. The fully methylated derivative is insoluble in sodium hydroxide, so that the solubility in alkali hydroxides appears to be due to the one free (phenolic?) hydroxyl group. From the evidence of the oxime we assume the presence of one carbonyl-group and one aldehydo-group in agreement with the results of Powell and Whittaker, who postulated two carbonyl- and one aldehydo-groupings in the  $C_{45}$  unit complex. The extended formula thus becomes:  $C_{16}H_{12}O(OCH_3)_2.OH.CO.CHO$ .

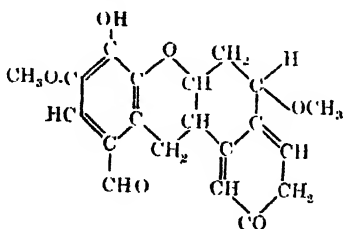
We have obtained a certain amount of evidence which makes it probable that the unassigned atom of oxygen exists in a pyrone ring and that the  $CO-CH_2$  group is also present. It will be remembered that Klason regards  $\alpha$ -lignin as derived from 2 molecules of coniferyl aldehyde, giving the following formula:



in which two aromatic nuclei are present. It seems impossible, with such a constitution, to explain the fact that on oxidation no aromatic products are obtained. The Klason formula closely resembles that of the anthocyanins, *e.g.* quercetin:



and the tetramethyl ether of this on oxidation with alkaline potassium permanganate yields veratric acid  $C_6H_3(COOH)(CH_3)_2$ , and probably phloroglucinol dimethyl ether. With metalignin on the other hand gentle oxidation, *e.g.* with dilute nitric acid, results in complete breakdown to oxalic and carbonic acids with the intermediate formation of non-aromatic nitro-compounds. We therefore prefer to regard metalignin as based upon hydro-aromatic nuclei in some such way as shown in the following ideal formula:





in which the two double linkages and the benzo-pyrone ring are readily opened up on oxidation tending to the disruption of the entire complex.

*Alkali resolution as a method for the estimation of lignin.*

Mehta [1925] has developed the following process for the quantitative estimation of lignin in wood. 5 g. dry wood in the form of fine sawdust are treated with 100 cc. of 4 % sodium hydroxide solution at 10 atmospheres pressure in a Pyrex glass tube for one hour. The lignin is said to be removed quantitatively as a sodium derivative leaving a pure  $\alpha$ -cellulose. The solution is acidified, and the precipitate dissolved in 95 % alcohol. The alcohol is removed and the residue dried to constant weight at 100°.

The figures arrived at, compared with those obtained on the same samples by the Willstätter process, show marked differences, as will be seen in Table II.

Table II.

	4 % NaOH	H <sub>2</sub> SO <sub>4</sub> .aq.	Difference
Lignum vitae ...	38.39	47.99	9.6 %
Teak ...	31.07	45.80	14.7
Larch ...	21.33	32.80	14.7
Spruce ...	17.80	30.55	12.7

The higher values obtained by the Willstätter method are ascribed to the presence of impurities and to combination with SO<sub>4</sub>H residues. The presence of impurities to the extent of 14.7 % is certainly high, and it is doubtful if the discrepancy can be due to this cause.

In the present work a quantitative examination was made of the spruce sawdust employed, to see if the difference in the values obtained by the two methods could be accounted for in any way. About 20 g. spruce wood, after extraction with benzene and alcohol and 5 % sodium hydroxide, were heated with 4 % sodium hydroxide in an autoclave for one hour at 10 atmospheres pressure. The sodium lignate was filtered off and the residual "cellulose" examined. This was at first red in colour, but quickly oxidised in the air to brown and finally to a dull green. After washing and drying this colour was still retained. It was never "snow white" or "exceptionally light" in colour as described by Mehta. Treatment of this "cellulose" with hydrochloric acid (d. 1.2) for twelve hours showed that it contained 5.4 % of lignin.

An examination was also made of the filtrate after precipitation of the lignin with hydrochloric acid. This was of a pale yellow colour which deepened to brown on the addition of alkali. The filtrate did not give a precipitate with a solution of calcium chloride, a result at variance with that of Powell and Whittaker. After neutralisation with ammonia, the liquid was evaporated almost to dryness on the water-bath, when a dark solid was obtained which was removed by extraction with alcohol. The alcoholic extract gave a brown

residue, soluble in water, pyridine, alcohol, and alkalis. It had an odour of  $\omega$ -hydroxy-methylfurfural.

A more exact quantitative experiment was also carried out: 5 g. spruce sawdust, after extraction for eight hours with benzene and alcohol and with water for four hours, were finally digested with a 5 % solution of sodium hydroxide for twelve hours to dissolve the hemicelluloses present. The purified sawdust was then autoclaved for one hour with 100 cc. of 4 % sodium hydroxide at 10 atmospheres pressure. The following values were obtained:

Metalignin (precipitated by acid) ... ..	18.6 %
Residue from filtrate (not precipitated by acid) ...	7.3
Lignin remaining in the cellulose ... ..	3.7
Total ... ..	<u>29.6</u>

In a further effort to ascertain the cause of the difference between the values obtained by Mehta's method and the hydrochloric acid method all the constituents of the wood were estimated by the methods of Dore [1920] (with the exception of the lignin). The following values were obtained (calculated on the original dry spruce sawdust, which contained 10 % of natural moisture):

Extraction with benzene ... ..	1.7 %
„ alcohol ... ..	1.6
„ water ... ..	2.5
„ sodium hydroxide (5 %) ... ..	3.8
Cellulose ... ..	55.0
Mannan ... ..	7.6
Galactan ... ..	0.1
Total ... ..	<u>72.3</u>

The difference (27.7 %) should be lignin, which agrees with the usual figure of 28–30 % found for spruce by direct estimation with hydrochloric acid. It is obvious, therefore, that Mehta has isolated only a fraction of the lignin present in the wood.

Part of the deficiency in total lignin-content can be accounted for by the retention of a portion of the lignin in the cellulose residue after heating with caustic soda, and the remainder is to be found in the water-soluble body present in the acid filtrate after precipitation of the lignin, which may be a decomposition product of lignin. The hemicellulose portion (3.8 %) was found to be separable into two parts, a part precipitated by acid, and a water-soluble fraction, precipitated by the addition of 95 % alcohol. These results are in agreement with those of O'Dwyer [1926], who found that there were two hemicelluloses present in beech-wood.

## SUMMARY.

1. By the action of 4% caustic soda at 10 atmospheres pressure for 1 hour on purified wood, a new type of lignin has been obtained and characterised, for which the name *metalignin* is proposed.

2. This lignin has the formula  $C_{20}H_{20}O_6$  with molecular weight 356. It melts at  $186^\circ$  and is soluble in a range of organic solvents, differing in these respects from lignins previously described.

3. The preparation of derivatives indicates the presence of one hydroxyl, two methoxyl and two carbonyl groups. Two double linkages are apparently present.

4. Metalignin agrees in composition with the  $\alpha$ -lignin of Klason [1920] existing as a sulphonic acid in bisulphite liquors.

5. The technique described in (1) above does not, as has been claimed [Mehta, 1925], give a quantitative separation of lignin.

One of us (E. C. B.-W.) desires to thank the Trustees of the Dixon Fund of London University for a grant towards the expenses of this investigation.

## REFERENCES.

- Beckmann, Liesche and Lehmann (1921). *Z. angew. Chem.* **34**, 285.  
— — — (1923). *Biochem. Z.* **139**, 491.  
Brady and Dunn (1913). *J. Chem. Soc.* **103**, 1613.  
Cross and Bevan (1882). *J. Chem. Soc.* **41**, 94.  
Dore (1920). *J. Ind. Eng. Chem.* **12**, 472, 476, 984.  
Dorée and Hall (1924). *J. Soc. Chem. Ind.* **43**, 257 t.  
Friedrich and Diwald (1925). *Monatsh. Chem.* **46**, 31.  
Fuchs (1926). *Die Chemie des Lignins*. (Berlin.)  
Hägglund (1924). *Cellulosechemie*, **5**, 81.  
Holmberg (1921). *Ber. deutsch. chem. Ges.* **54**, 2389, 2417.  
Klason (1920). *Ber. deutsch. chem. Ges.* **53**, 1864.  
— — — (1925). *Ber. deutsch. chem. Ges.* **58**, 375, 1761.  
Mehta (1925). *Biochem. J.* **19**, 979.  
O'Dwyer (1926). *Biochem. J.* **20**, 656.  
Powell and Whittaker (1924). *J. Chem. Soc.* **125**, 357.  
— — — (1925). *J. Chem. Soc.* **127**, 132.  
Urban (1926). *Cellulosechemie*, **7**, 73.  
Willstätter and Zechmeister (1913). *Ber. deutsch. chem. Ges.* **46**, 4201.

## **XLIII. STUDIES ON CALCIUM METABOLISM.**

### **I. THE ACTION OF THE PARATHYROID HORMONE ON THE CALCIUM CONTENT OF THE SERUM AND ON THE ABSORPTION AND EXCRETION OF CALCIUM.**

BY CORBET PAGE STEWART AND  
GEORGE HECTOR PERCIVAL (*Grocers' Company's Research Scholar*).

*From the Departments of Medical Chemistry and Pharmacology,  
University of Edinburgh.*

*(Received February 1st, 1927.)*

THE numerous references to the low calcium content of the serum in *tetania parathyreopriva*, and more particularly the recent work of Collip [1925] on the extraction and action of the parathyroid hormone, make it clear that one action, at any rate, of the parathyroid glands is to control the concentration of calcium in the blood. So much may be taken as definitely established, but of the mode in which this control is exercised little is known. Clearly the parathyroids may affect the absorption or excretion of calcium, or may exert some controlling action on the equilibria between the different forms of combination in which calcium exists in the blood and between the concentrations in the blood and in the tissues.

In view of the ease with which the low serum calcium of *tetania parathyreopriva* can be raised by administration of calcium salts, it appears unlikely that the parathyroids act by controlling calcium absorption. Some doubt exists, too, as to whether they have any effect on the excretion of calcium. Thus Kishi [1924] found that after parathyroidectomy there was increased urinary, but unchanged faecal, excretion of calcium; MacCallum and Voegtlin [1909] found an increased faecal excretion, and Greenwald and Gross [1925, 1] found the total excretion to be decreased. These last authors, too [1926], state that long-continued administration of parathyroid to normal dogs brings about an increased calcium excretion.

The third possibility is one accepted by Greenwald and Gross [1925, 2], who have suggested that the parathyroid hormone is, or is necessary for the preparation of, the substance which keeps in solution the large excess of  $\text{Ca}_3(\text{PO}_4)_2$  which Holt, La Mer and Chown [1925] have shown to be present in the blood. The excess of calcium excreted during long-continued administra-

tion of parathyroid they believe to be derived, ultimately, at any rate, from the bones.

In the work recorded in this paper we have aimed at obtaining some direct evidence as to the mode in which the parathyroid hormone exerts its action. The method of measuring the total excretion over a long period, first without and later with administration of parathyroid, is open to the objection that the differences usually observed are so slight as to be not far removed from the experimental error. They are not, moreover, easy of interpretation, for the faecal excretion is composed both of unabsorbed residues and of calcium re-excreted into the gut.

*The effect of haemorrhage on the serum calcium.*

Since many of our projected experiments necessitated the frequent drawing of blood samples and consequently involved considerable haemorrhage, it seemed advisable to test first the effect of haemorrhage itself on the calcium content of the serum.

Clark [1920], working with rabbits, found that extensive haemorrhage was followed by a considerable fall in the serum calcium, amounting to as much as 12 %.

We intended to use cats in our own experiments and therefore repeated Clark's work with these animals. To our surprise we found that, although the animal was bled freely at intervals of half an hour, there was no reduction in the serum calcium even at death. Numerous repetitions gave identical results; even with a loss of 60 % of the estimated total blood volume and a correspondingly large loss of haemoglobin, the serum calcium was never decreased by an amount greater than the experimental error of the method employed (that of Kramer and Tisdall [1921]). The results of these experiments are given in Table I.

The uniformity with which this effect was obtained determined us to repeat the experiments with the same species as was employed by Clark. In every case the results entirely confirmed Clark's observations.

There appears, therefore, to exist a definite species difference. The experimental conditions are identical in each series, the same anaesthetic being used, blood being taken at similar intervals and in similar amounts (with respect to the total blood volume), and the calcium estimations being carried out by the same method. We can offer no explanation of the difference, but it would seem to be in some obscure way connected with the susceptibility of the species to the action of the parathyroid hormone, for Greenwald and Gross [1926] have reported that, while cats, rabbits and rats are all more resistant than are dogs, rabbits and rats are even more resistant than are cats.

Table I. *The effect of haemorrhage on the serum calcium in the cat.*

No.	Weight of cat g.	Time min.	% haemoglobin *	Corpuscle volume % (haematocrit)	Mg. Ca per 100 cc. serum	Total haemorrhage cc.	% reduction in serum Ca	% reduction in haemoglobin
I	2800	0	66	35	9.90	—	—	—
		30	55	32	9.84	—	—	—
		60	53	29	9.87	—	—	—
		90	50	24	9.90	47	Nil	15
II	3000	0	65	31	10.3	—	—	—
		40	—	—	10.25	—	—	—
		80	—	—	10.3	—	—	—
		120	50	25	10.3	—	—	—
		150	—	—	10.4	—	—	—
		180	40	21	10.3	81	Nil	37.4
III	3000	0	70	—	9.5	—	—	—
		60	—	—	9.5	—	—	—
		120	—	—	9.5	—	—	—
		180	47	—	9.5	57	Nil	33
IV	2980	0	66	44	9.90	—	—	—
		40	—	—	9.90	—	—	—
		80	—	—	9.90	—	—	—
		120	—	—	9.90	—	—	—
		150	50	30	9.90	80	Nil	24
V	3000	0	67	—	11.0	—	—	—
		60	—	—	10.95	—	—	—
		120	—	—	10.95	—	—	—
		180	40	—	11.0	90	Nil	40
VI	3020	0	—	—	9.25	—	—	—
		60	—	—	9.25	—	—	—
		120	—	—	9.25	—	—	—
		180	—	—	9.25	89	Nil	—

Table II. *The effect of haemorrhage on the serum calcium in rabbits.*

No.	Weight of rabbit g.	Time min.	% haemoglobin *	Corpuscle volume % (haematocrit)	Mg. Ca per 100 cc. serum	Total haemorrhage cc.	% reduction in serum Ca	% reduction in haemoglobin
I	1500	0	62	30	12.90	—	—	—
		60	36	23	10.40	—	20	42
II	1000	0	53	32	11.20	—	—	—
		60	44	25	10.40	—	—	—
		100	38	23	9.50	—	14.4	28.5
III	2000	0	60	—	13.7	—	—	—
		30	52	—	13.2	—	—	—
		60	48	—	12.5	—	—	—
		90	40	—	12.4	44	8.6	33.3
IV	1950	0	60	27	11.5	—	—	—
		60	50	—	11.0	—	—	—
		120	20	15	10.0	33	10	66
V	1750	0	50	27	16.4	—	—	—
		75	40	21	13.6	30	17	20
VI	1690	0	60	28	11.9	—	—	—
		30	—	—	10.9	—	—	—
		90	—	—	10.5	—	—	—
		150	44	19	10.3	48	13.4	26.6

\* Haemoglobin estimated colorimetrically against human standard.

*The effect of parathyroid hormone on the blood serum of cats.*

The parathyroid hormone used in our earlier experiments was kindly presented to us by Dr McNee, who had obtained it from Dr Collip; later we employed the commercial preparation "Parathormone Lilly," extracted by Collip's method. Table III shows the effect of subcutaneous injections of these preparations on cats.

Table III. *Effect of Collip's parathyroid extract, given subcutaneously, on the serum calcium in cats.*

(0.5 cc. (10 units) parathyroid extract given after withdrawal of first blood sample.)

Exp.	Weight g.	Mg. Ca per 100 cc. serum		Increase
		Before injection	15 hr.	
I	2110	10.2	12.4	2.2
II	2440	9.7	11.9	2.2
III	2800	9.8	12.8	3.0
IV	3360	11.0	14.2	3.2
V	2800	10.4	14.1	3.7

10 units extract = amount necessary to produce in 15 hours a rise of 5 mg. per 100 cc. in a 2 kg. dog; therefore the extract is approximately half as active in cats as in dogs.

In every case it will be noted the serum calcium rose to an extent which, though less than would, presumably, have been observed in dogs, was well beyond the limits of experimental error and was undoubtedly real. The effect of intravenous injection of the hormone was next investigated. The cat was anaesthetised by means of paraldehyde and ether and a sample of blood having been withdrawn from the carotid, parathyroid hormone was given intravenously and thereafter blood was drawn at frequent intervals.

Table IV.

A. *Effect of intravenous injection of "Parathormone" in cats anaesthetised with paraldehyde and ether.*

Exp.	Weight of cat g.	Mg. Ca per 100 cc. serum before injection	"Parathormone" injected cc.	Mg. Ca per 100 cc. serum		
				30 min.	120 min.	160 min.
I	2210	10.1	0.5	10.9	11.8	11.1
II	2900	10.0	0.5	11.6	12.0	10.4
III	3300	10.4	1.0	10.9	12.0	11.6

B. *Effect of intravenous injection of "Parathormone" in decerebrate and pithed cats.*

Exp.	Weight of cat g.	Mg. Ca per 100 cc. serum before injection	"Parathormone" injected cc.	Mg. Ca per 100 cc. serum	
				45 min.	90 min.
I	3400	10.30	0.6	10.70	11.65
II	2890	9.6	0.6	11.70	12.00

The results of these experiments (Table IV) show that under these conditions the hormone exerts its action much more rapidly than when given hypodermically, the maximum rise in the serum calcium being attained in about 2 hours. The magnitude of the rise, however, is not greatly increased.

In Table IV (B) we also give the results of experiments in which parathyroid hormone was given intravenously to cats after decerebration which included removal of the pituitary gland, the spinal cord having also been destroyed. The rise in the serum calcium was of the same magnitude as that in anaesthetised animals (anaesthesia, as is shown *inter alia* by the results of the haemorrhage experiments, is without appreciable effect on the serum calcium). It is evident, therefore, that the effect of the parathyroid hormone is not exerted through the central nervous system.

We may remark at this point that *in vitro* we have observed a rapid inactivation of the hormone following its exposure to air (Table V).

Table V. *Effect of subcutaneous injection of Collip's parathyroid extract, which had been exposed to the air and kept at 37° for 24 hours, on the serum calcium in cats.*

Exp.	Weight of cat g.	Mg. Ca per 100 cc. serum before injection	Parathyroid extract injected (cc.)	Mg. Ca per 100 cc. serum 15 hr.
I	1980	9.5	0.5	9.5
II	2350	10.5	0.5	10.5

It is perhaps significant that the intravenous injection of sodium bicarbonate produces an effect exactly opposite to that of parathyroid. There is a rapid fall in the serum calcium reaching its maximum in some two hours, and thereafter returning to normal. Further, the two effects can be made to counterbalance each other exactly. As is shown in Table VI the simultaneous injection of sodium bicarbonate and of "Parathormone" (shown by control

Table VI.

A. *The effect of intravenous injection of sodium bicarbonate on the serum calcium in cats.*

Exp.	Weight of cat g.	Mg. Ca per 100 cc. serum before injection	NaHCO <sub>3</sub> injected g.	Mg. Ca per 100 cc. serum		
				30 min.	75 min.	100 min.
I	2110	10.8	0.8	10.0	9.4	8.8
II	2750	8.9	1.0	7.4	7.4	7.5

B. *The effect of simultaneous intravenous injection of NaHCO<sub>3</sub> and "Parathormone" on the serum calcium in cats.*

Exp.	Weight of cat g.	Mg. Ca per 100 cc. serum before injection	"Parathormone" (cc.) and NaHCO <sub>3</sub> (g.) injected	Mg. Ca per 100 cc. serum		
				30 min.	75 min.	100 min.
I	2750	8.8	0.7 cc. 1.0 g.	8.75	8.85	8.8
II	1870	10.2	0.7 cc. 1.0 g.	10.0	10.2	10.0



experiments to be active) leaves the serum calcium unaltered. Stewart and Haldane [1924] had previously found that oral administration of sodium bicarbonate caused a lowering of the human serum calcium (a result which we have confirmed), whereas oral administration of calcium chloride or of ammonium chloride, both of which produce an acidosis, caused a rise in the serum calcium.

These facts inevitably suggested the possibility that the action of "Parathormone" might be in some way connected with the neutrality-regulating mechanism. This idea, however, appears to have been partly disposed of by the recent work of Cantarow, Caven and Gordon [1926], who find that "Parathormone" is without effect on the  $\text{CO}_2$ -combining power of the blood.

Incidentally we may remark that oral administration of sodium bicarbonate affords two means of effecting a lowering of the blood calcium. By producing a decreased acidity of the intestinal contents it militates against the absorption of calcium; by bringing about an increased absorption of sodium, and hence an alkalosis, it may cause a direct lowering of the blood calcium. Following intravenous injection of sodium bicarbonate, the fall in the blood calcium can only be due to the alkalosis.

*The effect of "Parathormone" on the absorption of calcium.*

The direct measurement of the absorption of calcium is beset with many difficulties. Not only is the faecal output a combination of unabsorbed residues and of re-excreted calcium, the relative amounts of each being unknown, but the amount retained—which is all that can be measured—is obtained by difference from estimations of the total intake and total output, and is never more than a small fraction of either. The margin of error in such experiments is therefore considerable. Nor is it desirable to take the urinary excretion as an index of the amount of calcium absorption since it varies very considerably from day to day even under apparently standard conditions. The method of Bergeim [1926] undoubtedly gives a possible means of estimating absorption, but was published only after our experiments were under way. Indeed, excellent though it appears to be for many purposes, we believe it to be less satisfactory for our particular problem than the method we have employed. Briefly, our method consists in the complete removal of the alimentary canal distal to the oesophagus, *i.e.* the whole of that portion from which absorption may conceivably take place. If, under these conditions, the parathyroid hormone is able to exert its full effect on the serum calcium, it must mobilise calcium from some internal source or, possibly, act by controlling the rate of excretion. On the other hand, its inability to raise the serum calcium would indicate that normally the hormone draws on an external supply.

The technique of the experiments was as follows. Paraldehyde and ether were used to produce anaesthesia. The abdomen having been opened, the duodeno-jejunal junction was identified and the gut severed between two

ligatures at this point. The rectum was then drawn up out of the pelvis and cut across between ligatures close to the anus. The superior and inferior mesenteric arteries were ligated and the small and large intestine removed entirely. The duodenum was separated from the head of the pancreas, branches of the pancreatico-duodenal arteries being ligated. A series of ligatures were tied along the lesser and greater curvatures of the stomach from the pylorus to the cardia occluding the gastric vessels in the lesser omentum, and in the anterior layer of the greater omentum. The lower end of the oesophagus was ligated and cut across. The pylorus and first part of the duodenum were carefully separated from the portal vein, superior pancreatico-duodenal, splenic, and hepatic arteries, and the stomach and duodenum were removed by severing the omenta along the curvatures of the stomach. In this way the alimentary canal distal to the oesophagus was dispensed with, leaving the liver, spleen, and pancreas *in situ*, receiving an almost intact blood supply, that portion only of the portal circulation arising in the gut having been interfered with. The integrity of the arterial supply and venous return to these organs was in every case proved by the presence of arterial pulsation and by inspection of the venous flow. Where exclusion of liver, spleen and pancreas from the circulation was desired, the procedure was similar to that employed for evisceration alone, but it was unnecessary to separate the duodenum from the head of the pancreas. The hepatic artery and portal vein were ligated and cut in the free border of the lesser omentum, and the spleen and pancreas were removed entirely, after ligation of their respective arteries and veins.

At intervals blood was withdrawn through a cannula in the carotid artery, and injections were made through a cannula inserted in the external jugular vein. The animals were kept on artificial respiration and, despite the extensive surgical interference, their condition remained satisfactory throughout the experimental period. It was necessary, as a preliminary to the actual experiments, to ensure that in them the only variable condition was the presence or absence of added parathyroid hormone, since there were two other factors which might conceivably have caused an alteration in the calcium content of the blood. Firstly, it had to be shown that the operative shock was without effect in this direction. Secondly, in removing the whole of the alimentary canal, we had not only prevented absorption of calcium, but had also cut off one of the possible excretory routes. Control experiments in which evisceration was performed but no parathyroid hormone given showed that, over a period of three hours, the serum calcium remained absolutely unchanged. Whether the two factors are individually without effect, or whether their effects cancel one another was not determined, but was, for our purpose, a matter of no importance.

The results of a number of experiments on the effect of parathyroid hormone on the serum calcium of eviscerated cats are given in Table VII.

It will be noted that in every case the hormone produced a rise in the serum calcium, and comparison of these results with those given in Table IV shows

that the magnitude of the rise is as great in eviscerated as in normal animals. It seems fair, then, to conclude that the parathyroid hormone exerts its influence on the calcium content of the blood without drawing on external sources of calcium, *i.e.* without stimulating calcium absorption. Further, the liver, spleen and pancreas, have no special function as internal sources of calcium (though of course they may be used as reserves in the same way as other tissues) nor do they appear to influence the action of the hormone in any way.

Table VII.

*A. The effect of intravenous injection of "Parathormone" on the serum calcium of eviscerated cats.*

Exp.	Weight of cat g.	Mg. Ca per 100 cc. serum before injection	"Parathormone" injected (cc.)	Mg. Ca per 100 cc. serum	
				45 min.	90 min.
I	2760	9.0	0.7	9.8	11.2
II	2600	9.4	"	11.2	11.8
III	3500	8.4	"	11.4	10.2
IV	2990	9.6	"	10.6	10.8
V	3330	10.0	"	11.6	11.2
VI	3290	8.9	"	10.0	10.8

*B. The effect of evisceration and the removal of the liver, spleen and pancreas on the serum calcium in cats.*

Exp.	Weight of cat g.	Mg. Ca per 100 cc. serum before removal		Mg. Ca per 100 cc. serum	
				45 min.	90 min.
I	2860	9.80	Intestine, liver,	9.80	9.80
II	3950	10.50	spleen and pan-	10.45	10.45
III	3800	9.90	creas removed	9.85	9.90

*C. The effect of "Parathormone" on the serum calcium of cats in which the liver, spleen, pancreas, intestine and stomach have been removed.*

Exp.	Weight of cat g.	Mg. Ca per 100 cc. serum before injection	"Parathormone" injected (cc.)	Mg. Ca per 100 cc. serum	
				45 min.	90 min.
I	4050	9.80	0.6	11.00	11.35
II	2980	10.30	0.6	10.70	11.45
III	4010	9.90	0.6	10.60	11.10

*D. The effect of intravenous injection of sodium bicarbonate on the serum calcium in cats in which the liver, spleen, pancreas, stomach and intestine have been removed.*

Exp.	Weight of cat g.	Mg. Ca per 100 cc. serum before injection	NaHCO <sub>3</sub> injected g.	Mg. Ca per 100 cc. serum	
				45 min.	90 min.
I	3950	10.50	1	8.80	8.80
II	3100	9.50	1	8.30	7.90

*The effect of the parathyroid hormone on the excretion of calcium.*

If the parathyroid hormone acts by controlling the rate of calcium excretion, then it can only produce an increase in the serum calcium by diminishing the excretion; any increase in the excretion can only be a secondary effect due to the increased calcium concentration in the blood, the excess calcium being derived from the tissues (since increased absorption has been ruled out).

Calcium is undoubtedly excreted by the kidneys, but we desired to assure ourselves definitely that, as stated in the literature, it is also excreted through the epithelium of the large intestine. Further, if such intestinal excretion took place, we needed quantitative data. As has previously been stated, the calcium content of the large intestine represents both the unabsorbed residue and any excreted calcium; to estimate the latter directly it is necessary to exclude the former. To secure this end we adopted the following method.

The cat was anaesthetised by paraldehyde and ether, and placed on artificial respiration throughout the experimental period. Cannulae were inserted into the carotid artery and the external jugular vein. The abdomen having been opened, the small intestine was cut across between two ligatures close to the ileo-caecal valve. The large intestine was brought out and the pelvic colon cut across at its junction with the rectum, all bleeding points being ligated. A large bore cannula was inserted into the lip of the caecum. The isolated loop of large intestine was thoroughly washed out with warm distilled water through the cannula, the removal of solid and semi-solid material and mucous being aided by gently squeezing the bowel with the fingers. Washing was continued until a clear sample was obtained, and this was reserved for analysis. After every washing a final clear sample was collected separately and analysed. Only those experiments were considered in which this sample contained merely traces of calcium and so indicated that the washing had been complete.

Table VIII. *The excretion of calcium by the large intestine and by the kidney in the cat.*

Exp.	Weight of cat g.		Mg. Ca in intestinal washings	Mg. Ca in urine	Mg. Ca per 100 cc. serum		
					At start of exp.	10 min. after injection	At close of exp.
I	2900	1st 3 hours ...	0.68	0.21	—	—	—
		2nd 3 hours ...	0.66	0.14	—	—	—
II	2540	1st 3 hours ...	0.88	0.17	—	—	—
		2nd 3 hours ...	0.90	0.19	—	—	—
III	3010	1st 3 hours ...	0.33	0.22	—	—	—
		5 cc. 10 % $\text{CaCl}_2$ injected intravenously ...	—	—	10.2	22.0	15.0
		2nd 3 hours ...	4.27	0.25	—	—	—
IV	2700	1st 3 hours ...	0.68	0.16	—	—	—
		5 cc. 10 % $\text{CaCl}_2$ injected intravenously ...	—	—	—	—	—
		2nd 3 hours ...	14.0	0.15	—	—	—
V	2890	1st 3 hours ...	0.30	0.21	—	—	—
		5 cc. 10 % $\text{CaCl}_2$ injected intravenously ...	—	—	9.0	21.0	10.0
		2nd 3 hours ...	9.60	—	—	—	—

After this preliminary washing the isolated loop of intestine was replaced within the abdomen, with the two ends slightly protruding, so that any contents would not escape. The washing was repeated after three and after six hours. In a number of experiments a cannula was inserted into the bladder and samples of urine collected (with washing) at the same time as the intestine was irrigated. Table VIII, giving the results of experiments of this type, shows

that calcium was excreted into the gut and that this excretion occurred at a fairly constant rate. Further, it was usually greater than the urinary excretion for the same period.

It remained possible that the calcium found in the large intestine did not constitute a true excretion, but had merely been secreted along with the mucus. To test this point, we allowed a preliminary control period of three hours, then injected calcium salts intravenously, and again washed out the gut after a further three hours. After the injection of calcium salt the excretion of calcium into the intestine was enormously increased although there was no corresponding increase in the mucous secretion. The urine did not show any comparable increase in calcium content (Table VIII). It seems, then, that not only is calcium excreted by way of the large intestine, but that this is the main excretory route.

The effect of the parathyroid hormone was tested by a method exactly similar to that described above, an injection of the hormone being given at the end of a three-hour control period. That the hormone was active was shown in each case by the withdrawal of blood samples at intervals and estimation of the serum calcium. The results, set forth in Table IX, showed the hormone to have little or no effect on the intestinal excretion of calcium.

Table IX. *The effect of intravenous injection of "Parathormone" on the excretion of calcium by the large intestine in cats.*

Exp.	Weight of cat g.	Mg. Ca in large intestine after 1st 3 hr. period	Mg. Ca per 100 cc. serum before injection	"Parathor- mone" injected cc.	Mg. Ca per 100 cc. serum 60 min. after injection	Mg. Ca in large intestine after 2nd 3 hr. period	Mg. Ca per 100 cc. serum 3 hr. after injection
I	2790	0.74	10.6	2	11.4	2.0	12.0
II	2400	0.70	9.9	2	11.1	0.72	11.5
III	2300	0.64	9.8	2	11.0	0.80	11.7
IV	3050	0.60	10.0	2	11.2	0.60	11.8

In only one experiment was a definite increase obtained, but, on the other hand, in no case was there a decrease. The latter, as has been pointed out, is the important finding, and indicates clearly that the parathyroid hormone does not control the rate of excretion of calcium by way of the large intestine. The fact that little or no increase in the calcium excretion was observed in these experiments is not surprising in view of the small rise in the serum calcium produced by the parathyroid hormone compared with that following the injection of calcium salts.

Indeed when one considers the amount of calcium excreted during the control period, it seems hardly possible that the parathyroid hormone could produce the observed rise in the serum calcium by diminishing this amount. The average of a number of experiments shows that the total excretion of calcium in the control period of three hours is 0.8 mg., the greatest excretion observed being just over 1.0 mg. Now in all our experiments the serum calcium

has been at least 1.0 mg. per 100 cc. higher at the end of the three-hour period than at the beginning, and the net rise has usually been greater than this. The smallest cat used in these experiments weighed 2500 g., which means an approximate blood volume of 200 cc. and therefore at least 100 cc. of serum. Hence an increase of 1 mg. per 100 cc. in the serum calcium involves the mobilisation of 1.0 mg. calcium at least, an amount greater than the total excretion during the period in which this rise has taken place. Obviously then, control of the rate of excretion cannot be the main mode of action of the parathyroid hormone. When one adds the experimental finding that the intestinal excretion, which accounts for much more than half of the total, is not diminished at all, it follows that the hormone does not act by controlling the rate of calcium excretion.

#### DISCUSSION.

It is evident that the increase in the serum calcium following the administration of parathyroid extract is due to a withdrawal of calcium from the body tissues, for we have shown that the increase takes place without any diminution in the excretion, and without the possibility of any absorption from the alimentary canal.

Of the total serum calcium only about 60 % is readily diffusible through a collodion membrane [Cushny, 1920]. This readily diffusible fraction is apparently identical with Vines' "active calcium" [1924] which is precipitated by one equivalent of ammonium oxalate. Measurements of the ionic calcium [Neuhausen and Marshall, 1922] show that only 10–20 % of the total exists as ions. It is reasonable to suppose that the calcium mobilised by the parathyroid is readily diffusible, and that, therefore, the administration of parathyroid will be followed by a rise in the ratio of diffusible to total calcium in the serum. That such is actually the case has been stated by Vines [1924], and we have confirmed his statement [Stewart and Percival, 1927]. Several workers have shown that parathyroidectomy produces a relatively greater fall in the diffusible than in the non-diffusible calcium [Salvesen and Linder, 1924; Trendelenburg and Goebel, 1921; Moritz, 1925]. Hence it seems that on the amount of parathyroid hormone depends primarily the concentration of the readily diffusible calcium. This conclusion is to some extent in agreement with the suggestion of Greenwald and Gross [1925, 2] that the parathyroid hormone is, or is necessary for the production of, a substance (which Greenwald [1926] considers to resemble citric acid) capable of retaining in solution the excess  $\text{Ca}_3(\text{PO}_4)_2$  which Holt, La Mer and Chown [1925] have shown to be present in blood.

The calcium drawn into the blood when parathyroid is administered must come from the soft tissues or from the bones—or from both. Using whole animals, it seems impossible to gain any direct information as to which alternative is taken, except by long-continued administration of the hormone. with, perhaps, actual tissue analysis. In short experiments the quantities

involved are hopelessly small. Even in long ones the large number of analyses of intake and output, with the relatively small differences on which to found conclusions as to the quantity of calcium retained or lost, render the results of doubtful value. Greenwald [1926], however, in an experiment of this kind claims that the amount of calcium lost during the period in which parathyroid was administered could only have come from the bones. He points out, however, that the blood may have drawn its extra calcium from the soft tissues which thus made up their supply at the expense of the bones. The rapidity with which parathyroid produces a rise in the blood calcium suggests, indeed, that the soft tissues form the primary source of supply.

We are at present engaged in a series of experiments by means of which we hope to gain some further information as to the immediate source of the calcium mobilised by the parathyroid hormone.

#### SUMMARY.

1. Extensive haemorrhage causes a lowering of the serum calcium in rabbits but not in cats. Rabbits are less susceptible to the action of parathyroid extract than are cats.

2. Parathyroid extract ("Parathormone") raised the serum calcium of cats when injected subcutaneously, and more rapidly when injected intravenously. The action is prevented by the simultaneous injection of sodium bicarbonate.

3. Since the parathyroid hormone can exert its full action on the serum calcium even after complete removal of the alimentary canal, it is concluded that the action does not consist in controlling the rate of calcium absorption.

4. The large intestine provides the main excretory route for calcium.

5. Following administration of parathyroid, and while the serum calcium is high, there is no diminution in the excretion of calcium. The parathyroids, therefore, do not act by controlling the rate of excretion.

6. The liver, spleen, pancreas, pituitary, thyroid, and central nervous system appear to have no special function in connection with the effect of parathyroid on the blood calcium.

7. It is considered that the parathyroid hormone controls the distribution of calcium between the blood and the tissues by regulating the proportion of the total serum calcium which is readily diffusible.

The thanks of one of us (C. P. S.) are tendered to the Medical Research Council, from whom he is in receipt of a personal grant. The expenses of the work have been defrayed by grants from the Earl of Moray Research Fund of this University and from the Grocers' Company.

## REFERENCES.

- Bergeim (1926). *J. Biol. Chem.* **70**, 29.  
Cantarow, Caven and Gordon (1926). *Arch. Int. Med.* **38**, 502.  
Clark (1920). *J. Biol. Chem.* **43**, 89.  
Collip (1925). *J. Biol. Chem.* **63**, 395.  
Cushny (1920). *J. Physiol.* **53**, 391.  
Greenwald (1926). *J. Biol. Chem.* **67**, 1.  
Greenwald and Gross (1925, 1). *J. Biol. Chem.* **66**, 185.  
—— — (1925, 2). *J. Biol. Chem.* **66**, 217.  
—— — (1926). *J. Biol. Chem.* **68**, 325.  
Holt, La Mer and Chown (1925). *J. Biol. Chem.* **64**, 509, 567, 579.  
Kishi (1924). *Ber. ges. Physiol.* **24**, 364 (abstract).  
Kramer and Tisdall (1921). *J. Biol. Chem.* **47**, 475.  
MacCallum and Voegtlin (1909). *J. Exp. Med.* **11**, 118.  
Moritz (1925). *J. Biol. Chem.* **66**, 343.  
Neuhausen and Marshall (1922). *J. Biol. Chem.* **53**, 365.  
Salvesen and Linder (1924). *J. Biol. Chem.* **58**, 617, 635.  
Stewart and Haldane (1924). *Biochem. J.* **18**, 855.  
Stewart and Percival (1927). *Quart. J. Med.* (In the Press.)  
Trendelenburg and Goebel (1921). *Arch. exp. Path. Pharm.* **89**, 171.  
Vines (1924). *The Parathyroid Glands in Relation to Disease.*



## XLIV. ON LIVER AMYLASE.

By GEORGE SHARP EADIE.

*From the Biochemical Laboratory, Cambridge.*

*(Received February 4th, 1927.)*

IN view of certain peculiarities in the relation of the velocity of hydrolysis of starch by the amylase of germinated barley to its concentration [Eadie, 1926], it was decided to investigate the relations which hold in the case of liver amylase and glycogen, in the hope of throwing more light on the physiology of glycogen in the liver and the regulation of carbohydrate metabolism. Early in this work the disconcerting fact was disclosed that the optimum hydrogen ion concentration varied with the preparation used, and was now at  $p_H$  7 and now at  $p_H$  6. Earlier workers have uniformly stated it to be about  $p_H$  7 (see for example, Holmbergh [1923], who gives references to the earlier papers), although careful search of the literature shows that this has not always been justified by their results. During the course of these experiments a paper by Davenport [1926] was published in which this peculiarity was noticed when liver amylase acted on starch.

Since the liver preparations used contained more or less blood, it seemed possible that there were really two or more amylases present. The amylase of the blood [Euler, 1922] is probably derived for the most part from the pancreas, and pancreatic amylase has an optimum about  $p_H$  7. This suggested that the more alkaline optimum in the case of the liver preparations was due to amylase derived from the blood present in the liver, and that the liver amylase itself was responsible for the more acid optimum.

### METHODS.

The extent of hydrolysis was estimated by determining the amount of sugar produced, using the method of Shaffer and Hartmann. Reducing power was calculated as glucose. The objections raised by Visscher [1926] to the use of this method in the presence of phosphate buffers apparently do not apply when the concentration of phosphate is as low as in these experiments [Davenport, 1926].

The liver was prepared by a method similar to that described by Holmbergh [1923]; it was thoroughly ground, then treated twice with acetone, once with a mixture of equal parts of acetone and ether, and finally twice with ether. The quantities and times recommended by Holmbergh were not strictly followed. After drying overnight the powder thus obtained was extracted for several days with 50 % glycerol, filtered and dialysed against running water for about five hours. The resultant solution was frequently found to be unstable, but it could always be used for 24 hours.

Clark and Lubs' phosphate buffers were used and checked electrometrically.

Liver from various sources was tried, but the most active preparations came from the rat rather than from the pig or sheep.

The glycogen used was prepared by Pflüger's method, for the most part from rabbit liver, but in a few experiments that from mussels was used. No difference was found between the glycogens from these two sources.

During hydrolysis the reacting solutions were kept in a thermostat at 37°.

#### EFFECT OF PERFUSION ON RAT LIVER<sup>1</sup>.

A rat liver was treated by the method just outlined after washing off the blood but without perfusion, and its activity tested in the following way.

Into each of five flasks were placed 5 cc. of 1.5 % glycogen solution, 0.5 cc. of 2.5 % sodium chloride solution, 5 cc. buffer (of varying  $p_H$ ) and 15 cc. water. These were warmed to 37°, and 5 cc. of the enzyme solution were added to each. Samples removed immediately after mixing showed the absence of sugar. Samples were taken in 2, 4 and 24 hours. The results for the 4-hour samples are shown in Fig. 1 (continuous line), and those for the other two periods are in substantial agreement with these. An almost identical curve was obtained with sheep's liver. It will be noted that there are two maxima, one about  $p_H$  6 and another about  $p_H$  6.8 or 6.9.

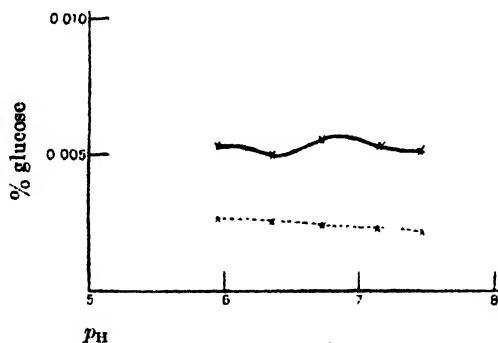


Fig. 1. Effect of perfusion.

——— Unperfused liver.

---- Perfused liver. The figures on the ordinate refer to the unperfused liver.

For the actual values for the perfused liver see text.

Another rat was then taken and perfused for about an hour with warmed Ringer's solution. After this time the liver showed no traces of blood. Treated in an exactly similar way, it gave the results shown below.

$p_H$ : 5.95, 6.35, 6.72, 7.14, 7.41.

Glucose % after 5½ hours: 0.0525, 0.0510, 0.0480, 0.0465, 0.0440.

These results are plotted in Fig. 1 (dotted line) on the same scale as the other curve, but to prevent the curves overlapping, another base line is used.

<sup>1</sup> I am indebted to Mrs Holmes for her kindness in perfusing these livers.

The figures in the margin should each be increased by 0.0025 to apply to this curve.

Here the maximum between 6.7 and 7.1 has disappeared. Evidently the perfusion has washed out the blood and with it an amylase having its optimum about the neutral point.

Since the rat liver is rather small, I endeavoured to find a method for freeing from blood livers from the slaughter-house. For this purpose a quantity of pig's liver was finely ground, stirred up with water, centrifuged and again treated with water, the process being repeated four times.

After being treated in the usual way this preparation was allowed to act on glycogen under the same conditions as before. After 5 hours' hydrolysis the following amounts of sugar were found:

$p_H$ : 5.95, 6.35, 6.72, 7.14, 7.41.

Glucose %: 0.0016, 0.0028, 0.0032, 0.0040, 0.0020.

This was clearly unsuccessful.

Another plan was then tried based on that used by Thunberg for washing muscle. The ground liver was placed in a tall glass cylinder covered with gauze through which a glass tube passed leading to the bottom of the cylinder. Tap water was run through the glass tube until the water coming away was clear. The residue was treated in the usual way and the extract allowed to act as before. In 4½ hours the amounts of sugar produced were:

$p_H$ : 5.95, 6.35, 6.72, 7.14, 7.41.

Glucose %: 0.0056, 0.0050, 0.0062, 0.0056, 0.0053.

There has been a shift of the optimum towards the acid side here but not so completely as in the case of the perfused rat liver. Obviously some blood amylase is still present although in relatively less amount.

Since the rat liver was so much more active and so much easier to wash free from blood, it was decided to continue with it. Lesser [1920] considers that washing of the liver takes out some of the liver amylase, and I find it associated with considerable loss of activity. Since the pig's liver was not very active to begin with there could be very little activity left after sufficient washing to free it from blood. .

It should be noted here that this change in optimum  $p_H$  is not of the kind described by Michaelis and Pechstein [1914] among others in the case of ptyalin, where it varies with the salt present in the mixture, for in all cases here the same amounts of phosphate and sodium chloride were used. Moreover, the presence of two optima in certain experiments with unwashed liver could not be explained in this way.

#### INFLUENCE OF SUBSTRATE CONCENTRATION.

Fig. 2 shows the amounts of sugar formed in an hour under the following conditions. Six flasks were prepared containing 0.5 cc. of 2.5 % sodium chloride solution, 5 cc. phosphate buffer of  $p_H$  5.95, varying quantities of

1.5 % glycogen solution and water to make a total volume of 25.5 cc. After being warmed to 37°, 5 cc. of enzyme solution were added to each.

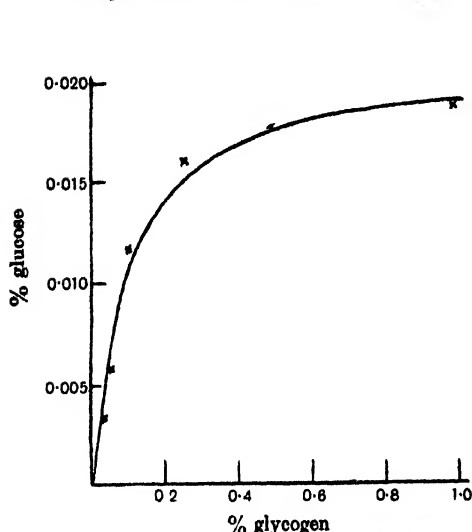


Fig. 2. Effect of substrate concentration.

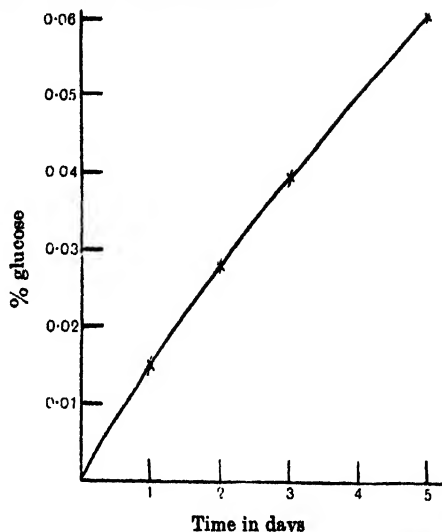


Fig. 3. Showing initial stages of hydrolysis.

That the rate of reduction is practically linear over this range is shown by Fig. 3, which refers to a solution of a similar composition containing 0.45 % of glycogen. During the time covered by the curve 0.06 % of glucose was produced, *i.e.* 10 to 15 % of the amount for total hydrolysis. This is about the extent of hydrolysis in the experiment illustrated in Fig. 2. Any deviation from the linear relation is likely to produce results which are too low in the case of the lower concentrations of glycogen and (relatively) too high in the case of the higher concentrations.

The smooth curve in Fig. 2 is based on the theory proposed by Michaelis and Menten [1913], who deduce from it the equation:

$$v = k \cdot \frac{S}{S + K_s}$$

Here  $v$  is the velocity,  $S$  the substrate concentration,  $K_s$  the "affinity constant" or Michaelis constant, and  $k$  is another constant. The curve in Fig. 2 is plotted from this equation, putting  $K_s = 0.095$  and  $k = 0.00021$ . This value for the affinity constant is of the same order of magnitude as those obtained by Michaelis in the paper quoted.

It will be seen that the agreement of the values found with those predicted from theory is within the limits of experimental error. Closer examination reveals that the first two experimental points lie below the curve and the three following above it, which might indicate a constant trend. I think that this is due to the fact that the velocity (as mentioned above) falls off somewhat with time, and affects the points corresponding to the smaller concentrations first. I have tried several other types of curve, but no other seems to fit the results nearly so well.

These results therefore differ markedly from those obtained with starch and the amylase of germinated barley, and resemble those obtained with such a system as sucrose and invertase.

An attempt was made to do this experiment with a preparation made from unwashed liver. The curve obtained resembled this one somewhat, but, as might be expected, was rather more complex and did not fit exactly any well-known type of curve.

#### EFFECT OF SALT CONCENTRATION.

Since it is probable that most previous workers have used mixtures of blood and liver amylase to investigate this point, it was thought worth while to repeat the experiment.

Flasks were set up each containing 5 cc. of 1.5 % glycogen solution, varying quantities of 0.30 % sodium chloride solution, and water to make 20 cc. 5 cc. of enzyme solution were added to each in the manner described above. The concentration of glycogen thus was 0.25 %. The sugar formed in one hour was estimated and the results are given in Fig. 4.

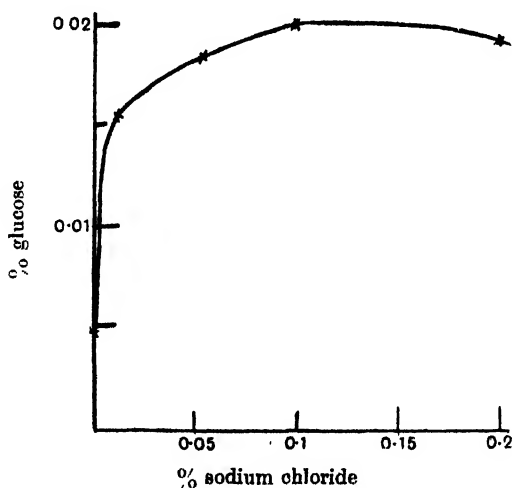


Fig. 4. Effect of salt concentration.

It will be seen that this amylase resembles closely the other amylases of animal origin in being extremely sensitive to the presence of sodium chloride. The optimum is apparently about 0.1 %.

#### EFFECT OF ADRENALINE.

These experiments were done in the spring of 1926. Since that time two other contributions to the subject have been made. It will be recalled that Langfeldt [1921] claimed that adrenaline not only increased the activity of the liver amylase, but also altered its optimum hydrogen ion concentration.

Working with liver amylase and glycogen I was not able to find any alteration in the optimum  $p_H$  in the presence of adrenaline. There was, however, increased activity, but this disappeared when the concentration of sodium chloride in the controls and in the solutions containing adrenaline was made equal. Commercial preparations of adrenaline contain considerable amounts of sodium chloride.

Visscher [1926] using glycogen as a substrate found no alteration of the optimum  $p_H$ , but his enzyme was evidently badly contaminated with blood amylase, and he did not discover the chloride effect. Later, Davenport [1926] reported that with starch as a substrate adrenaline had no effect, apart from the sodium chloride contained in its preparations. Davenport used several enzyme preparations, some of which contained blood amylase in large quantity while others did not. I have found a similar variation in the blood amylase content in preparations made by Holmbergh's method; the method used by Davenport evidently gives similar results.

It appears therefore that Langfeldt's claims are not substantiated.

#### DISCUSSION.

In view of the failure of earlier workers to recognise that their preparations of liver amylase might be badly contaminated by that from the blood, a good deal of their work requires revision.

One of the most detailed studies of the enzyme is that by Holmbergh [1923]. He states that the optimum  $p_H$  in the presence of sodium chloride and phosphate buffer is 6.9. This indicates that his preparation contained so much blood amylase that its activity overshadowed that of the liver; consequently his estimates of the quantity of amylase in the liver are probably much too high. Similar suspicion also attaches to other estimates for in no case is there proof that liver amylase alone is being estimated. Indeed, to estimate the amount of liver amylase is a matter of some difficulty: no method of washing out the blood seems satisfactory, because, as Lesser and Kerner [1920] have shown, thorough perfusion also washes out some of the true liver amylase.

My experiments have also some bearing on the question of glycogenolysis. For the problem of how amylase and glycogen can exist together in the liver cell, several solutions have been offered. One of these is that, to quote Macleod [1922], "glycogenolysis in the liver cell could be set up by the local production of a certain amount of acid. Such a liberation of free acid could be brought about by a curtailment in the arterial blood supply of the hepatic cell. . . " and vasoconstriction either from adrenaline or nervous stimulation is suggested as a probable cause. The argument includes the remark that "diastatic enzymes are particularly susceptible to the reaction ( $c_H$ ) of their environment, a very slight degree of acidity favouring and a trace of alkalinity markedly depressing their activities." It is apparently assumed that at the normal  $p_H$  of the liver cell the enzyme is inactive, and that its activity enormously

increases with the degree of acidity. It seems unlikely that a physiological process such as vasoconstriction would lead to such a change as from  $p_H$  7.4 to 6. Yet we see that the enzyme is by no means inactive at the former  $p_H$ , and in changing to the latter its activity increases by less than 25 % (cf. Fig. 1). A change from  $p_H$  7.4 to 6.7, which is probably much closer to changes occurring in the living cell, increases the activity by about 10 %.

Since the rate of hydrolysis is very nearly constant over a considerable period at the beginning, the figures from which these calculations are made give a pretty close measure of the initial rates, and may be used in place of them without any error which would affect this argument in the slightest.

The liver amylase itself is either not an active enzyme compared with that of the blood, or is present in very small concentrations, for the presence of blood in the organ from which the preparation is made is able to mask it almost completely. It is very much weaker than that of the pancreas. These facts, in connection with the flatness of the activity- $p_H$  curve, render the explanation quoted above much less probable than it appeared to be in the absence of more detailed knowledge of the enzyme.

Lesser [Lesser and Kerner, 1920; Lesser, 1920, 1921] has advocated another view, viz. that the glycogen and amylase are, as it were, "locked up" in different compartments of the cell. This explanation unfortunately adds little to our knowledge because it suggests no mechanism whereby the two are kept separate. Lesser finds a loss of activity on perfusion which he attributes to the washing-out of liver amylase only, for he maintains, on the basis of experiments by himself and by Fröhlich and Pollak, that the amylase of the blood can have no action on the liver glycogen. He reports [1921] an experiment in which he perfused livers with solutions of varying  $p_H$  and measured the hourly output of glucose. The figures for the first hour's perfusion show maximal amounts at  $p_H$  7.44 and 5.66. The figures for the second, third and fourth hours show maxima at 6.45. (There was no solution of  $p_H$  intermediate between 5.66 and 6.45.) These values refer of course to the perfusion fluid and are not to be taken as indicating very accurately the conditions under which the actual hydrolysis occurs, although they must influence it to a great extent and one which becomes greater as time goes on.

These facts would be simply explained if we admit that the blood amylase is active during the first hour, but is washed out by perfusion much more rapidly than the liver amylase. If this be admitted, I suggest that it is quite possible that blood amylase may act on glycogen under physiological conditions.

#### SUMMARY.

1. The optimum  $c_H$  for liver amylase is shown to be at least as high as that indicated by  $p_H$  6, and the previously accepted value is attributed to the amylase of the blood.
2. The effect of substrate concentration on the activity has been determined and shown to be in accordance with the theory of Michaelis.

3. The effect of sodium chloride is shown to be similar to that found with other amylases of animal origin, and the effect of adrenaline solutions is found to be due solely to the sodium chloride contained in them.

4. The bearing of these experiments on the problem of glycogenolysis in the liver is discussed.

I should like to acknowledge my indebtedness to Sir F. G. Hopkins and Mr J. B. S. Haldane for much helpful criticism.

#### REFERENCES.

- Davenport (1926). *J. Biol. Chem.* **70**, 625.  
Eadie (1926). *Biochem. J.* **20**, 1016.  
Euler (1922). *Chemie der Enzyme*, II. 1.  
Holmbergh (1923). *Z. physiol. Chem.* **134**, 68.  
Langfeldt (1921). *J. Biol. Chem.* **46**, 391.  
Lesser (1920). *Biochem. Z.* **102**, 304.  
—— (1921). *Biochem. Z.* **119**, 108.  
Lesser and Kerner (1920). *Biochem. Z.* **102**, 284.  
Macleod (1922). *Physiology and Biochemistry in Modern Medicine*.  
Michaelis and Menten (1913). *Biochem. Z.* **40**, 333.  
Michaelis and Pechstein (1914). *Biochem. Z.* **59**, 77.  
Visscher (1926). *J. Biol. Chem.* **69**, 3.



# **XLV. THE BEHAVIOUR OF WHOLE BLOOD TOWARDS MALTOSE *IN VITRO*.**

BY ALEXANDER HYND AND MARJORIE GIFFEN MACFARLANE.

*From the Physiology Department, University of St Andrews.*

*(Received February 23rd, 1927.)*

MALTOSE is the only disaccharide which, on subcutaneous injection, has been found capable of alleviating the symptoms caused by insulin. As has been shown by Herring, Irvine and Macleod [1924], its action, in this respect, in the case of mice is almost as efficient as that of glucose or mannose, but with larger animals, such as rabbits, cats or dogs, it is less effective. Even with mice, it exercises its action more slowly than glucose and this would suggest that maltose requires to undergo hydrolysis before it can counteract the action of insulin. Further, the inequality in the rates of hydrolysis of maltose might account for the difference in the results obtained in different species of animals. Support for this view seemed to be furnished by the results of investigations on the maltase content of blood-serum reported by Compton [1921]. This worker found that the maltase content of the blood was not the same in all animals and he divided mammals into two groups, according to whether they had or had not maltase in their blood. Unfortunately, Compton did not include the mouse amongst the animals he investigated, and the experiments now recorded were undertaken, initially at least, to determine whether the greater efficiency of maltose in relieving mice from the effects of an injection of insulin could be correlated with a greater maltase content of the blood in this animal than in larger animals such as the rabbit or dog.

## **EXPERIMENTAL.**

Our experimental procedure was very similar to that described by Compton except that whole blood was used in place of serum, and Wood-Ost's method for the sugar determinations was employed instead of Bertrand's. One of us (A. H.) is responsible for the animal experiments necessary for the research, while the other (M. G. M.) carried out the greater part of the chemical analyses. All the apparatus used (flasks, pipettes, etc.) was sterilised, and the sugar solutions (4-6 %) were prepared with water previously saturated with toluene. Blood from the following eight species of animals, namely, mouse, rat, guinea-pig, rabbit, kitten, pig, sheep and ox, was examined. The smaller animals, mouse, rat, and guinea-pig, were chloroformed and bled from the neck: the kitten was also anaesthetised and blood taken from either the carotid artery or jugular vein; the rabbit's blood was obtained from the marginal ear vein, while blood of the pig, sheep and ox was procured directly from the

After 5 cc. of the maltose solution and 1 cc. of blood had been incubated at 50° for 24 hours, 43 cc. saturated sodium sulphate solution were added and the mixture brought to the boil. After cooling, 1 cc. of "dialysed iron" was added to complete the precipitation of the blood proteins. Ten cc. portions of the resulting clear filtrate, which is exactly ten times more dilute than the original maltose solution, were used to determine the sugar content. Two control experiments were always carried out simultaneously. In one, 5 cc. of the maltose solution and 1 cc. of blood were mixed and immediately heated to boiling to inactivate all enzymes present. Thereafter the mixture was incubated at 50° for 24 hours and the sugar estimated as described above. In the other, 5 cc. of an approximately equivalent glucose solution and 1 cc. of blood were incubated at 50° for 24 hours. and the glucose content then determined and compared with the theoretical amount.

### Table of Results.

E = concentration of glucose after 24 hr. at 50° with blood.

Animal				Temp.	A	B	C	D	E
				°	%	%	%	%	%
1.	Rat (normal)	...	...	37	-616	-629	-646	—	—
2.	"	...	...	"	-616	-635	-618	—	—
3.	"	...	...	50	-612	-632	-629	-608	-584
4.	"	...	...	"	-612	-620	-616	-495	-466
5.	Guinea-pig (normal)	...	...	"	-612	-560	-616	-495	-460
6.	"	...	...	"	-612	-568	-600	-495	-461
7.	Mouse (high carbohydrate diet)	...	...	"	-328	-352	-336	-388	-388
8.	" (low carbohydrate diet)	...	...	"	-328	-352	-352	-388	-396
9.	Rabbit (normal)	...	...	"	-593	-593	-600	-495	-455
10.	" (after daily intravenous injection of maltose for a week)	...	...	"	-593	-584	-585	-388	-379
11.	Kitten (excessive carbohydrate diet)	...	...	"	-616	-623	-616	-577	-583
12.	" ( " protein diet)	...	...	"	-616	-632	-638	-577	-585
13.	" ( " fat diet)	...	...	"	-616	-633	-637	-577	-584
14.	Ox (normal)	...	...	"	-609	-625	-610	-516	-525
15.	"	...	...	"	-609	-615	-612	-516	-536
16.	Sheep (normal)	...	...	"	-595	-552	-565	-516	-519
17.	"	...	...	"	-595	-591	-578	-516	-523
18.	Pig (normal)	...	...	"	-365	-724	-355	-445	-446
19.	"	...	...	37	-365	-558	-364	-445	-428

Complete hydrolysis of the filtrates obtained in the maltose experiments with both blood and "boiled" blood was also carried out as follows. To 20 cc. of filtrate 2 cc. concentrated hydrochloric acid were added, and the mixture boiled gently under a reflux condenser for half an hour. The solution was then neutralised with the *exact* amount of sodium carbonate solution, the total volume adjusted to 50 cc., and portions used for analysis. The results of these experiments need not be reported in detail, as in every case values approximating closely to the theoretical were obtained.

Further control experiments were undertaken in which 1 cc. of an extract of commercial maltase was used in place of blood. In the first of these experiments 1 cc. of extract was added to 5 cc. of the maltose solution: in the second a trace of potassium oxalate was also added to the mixture, while the third was heated to boiling immediately on mixing the maltose solution with the enzyme extract. All three were then incubated for 24 hours at 50°, and examined exactly as described for the experiments with blood. The following results were obtained.

			Reducing power expressed as glucose		% of maltose hydrolysed
			Initial	After 24 hr. at 50°	
1.	Maltose + enzyme extract	...	0.616 %	1.071 %	87 %
2.	" + " + oxalate	...	"	1.045	85
3.	" + boiled enzyme extract	...	"	0.694	—

The  $p_H$  of the above three mixtures as determined by the colorimetric method was identical, namely 6.7. As this method could not be applied directly to the experiments in which blood was used, the  $p_H$  of a mixture containing 5 volumes of the maltose solution and 1 volume of a phosphate buffer of  $p_H$  7.4 was determined and found to be 6.9.

#### DISCUSSION.

From the results tabulated above, it is evident that during the incubation no appreciable loss of glucose took place in any of our experiments, as a close agreement exists in every case between the values in columns D and E. Consequently the glycolytic action of the blood was a negligible factor in these experiments. Further, the values in columns A, B and C also exhibit a close agreement in the case of all the animals used except the pig. Maltase would thus appear to be absent from the blood of these animals, or only present in amounts too small to be detected by the method employed. At the same time, it is to be noted that, under identical experimental conditions, in the case of the pig, while the value for C (0.355 %) agreed with that of A (0.365 %), B was increased to an amount (0.724 %) corresponding to almost 100 % hydrolysis of the maltose used. This result is in agreement with that of other workers as to the high maltase activity of pig's blood, and also indicates that our experimental conditions were suitable for the enzyme action to proceed.

Although the  $p_H$  of the reaction mixtures was not determined, it was assumed, in view of the control experiment with the phosphate buffer referred to above, that the  $p_H$  of each mixture would be in the neighbourhood of 6.7-6.9, and as the experiments were conducted at 50°, the conditions employed were, according to the researches of Compton [1915, 1924], the optimum both as regards temperature and reaction. Additional proof of this is afforded by the result of the experiments in which an extract of a commercial sample of maltase was used. In this case 87 % of the maltose was hydrolysed. As with the addition of a trace of potassium oxalate 85 % was hydrolysed, the oxalate used to prevent clotting in the experiments with blood would exert no inhibiting action on maltase.

Consequently, the negative results obtained in the case of the mouse, rat, guinea-pig, rabbit, kitten, ox and sheep indicate that maltase is lacking from the blood of these animals. This is not altogether in agreement with the observations of other workers. Thus, while the rabbit, guinea-pig and kitten fall in his "non-maltase" group, the rat and sheep belong to Compton's "maltase" group, but Doxiades [1911], on the other hand, using a polarimetric method, gives figures indicating that rabbit's serum has a maltase activity as great as that of the sheep or dog. Bial [1893], also, at an earlier date, claimed to have demonstrated the presence of maltase in human blood by the isolation of glucose phenylosazone, although it is to be noted that no change in the polarimetric or reducing value of the solutions was detected. The discrepancy between the results of other workers and those now recorded does not appear to be due to the substitution of whole blood for blood-serum, for an experiment with pig's serum gave a much lower percentage of maltose hydrolysed (50 %) than a similar experiment with whole blood (100 %). It seems likely that the discordant results depend partly on different methods being adopted to detect the enzyme action and partly on a larger proportion of serum being employed by earlier workers. Taking the results as a whole, they do not support Compton's classification.

In our experiments we failed to detect the presence of maltase in the blood of any of the animals employed except the pig: and we did not succeed in our attempts to affect the content of blood maltase by different diets in the case of mice and kittens or by a daily intravenous injection of maltose in the case of a rabbit. Our results point to there being no distinct difference in the activity of maltase in the blood of the mouse, as compared with that of the rabbit. Consequently it follows that if maltose counteracts the effects of insulin only after hydrolysis, such hydrolysis does not occur in the blood. As it is known that an insulinised mouse responds more readily to an injection of maltose than does an insulinised rabbit, it is possible that hydrolysis is effected by an enzyme present in the tissues.

On the other hand, it may be noted that maltose, being a glucose  $\alpha$ -glucoside, differs from other sugars (save glucose) so far employed to alleviate the symptoms caused by insulin, in having in its molecule, at one and the same

time, a *reducing group* and a *complete glucose configuration*. As the combined effect of these two factors may account for the efficiency of a compound sugar in counteracting the effect of insulin, experiments with cellobiose and gentiobiose in this connection would be of great interest. However, until further work can be carried out, it must remain an open question as to whether the efficiency of maltose in alleviating the symptoms resulting from insulin administration depends on the constitution of the compound or on its hydrolysis to glucose. In the latter case, the splitting up of the disaccharide is not accomplished by the blood maltase, but probably takes place in the tissue cells.

## REFERENCES.

- Bial (1893). *Pflüger's Arch.* **53**, 156; **54**, 72.  
Compton (1915). *Proc. Roy. Soc. Lond. B*, **88**, 258, 408.  
—— (1921). *Biochem. J.* **15**, 681.  
—— (1924). *Biochem. J.* **18**, 173.  
Doxiades (1911). *Biochem. Z.* **32**, 310.  
Herring, Irvine and Macleod (1924). *Biochem. J.* **18**, 1023.

# XLVI. STUDIES IN THE METABOLISM OF TISSUES GROWING *IN VITRO*.

## I. AMMONIA AND UREA PRODUCTION BY KIDNEY.

BY BARBARA ELIZABETH HOLMES AND ELSIE WATCHORN.

*From the Biochemical Laboratory, Cambridge.*

*(Received February 28th, 1927.)*

THE present work is an attempt to approach the study of the metabolism of some growing mammalian tissues. While much work has been carried out on the adult surviving or functioning organ by such methods as perfusion experiments, extirpation of an organ, etc., we know practically nothing of any chemical changes due actually to growth of any particular embryonic organ in the mammal. Probably the only way to study such changes is by means of the technique of tissue culture, where growth takes place *in vitro*. It is through this technique, suitably adapted to our needs, that we have endeavoured to attack the problem. In this communication the changes in ammonia and urea content of the embryo rat kidney are discussed, together with a note on the brain.

### *Technique.*

A technique had to be devised which would give us sufficient material for analytical treatment; it was also necessary to use a liquid culture medium. The routine finally adopted is as follows.

A pregnant doe is anaesthetised and the two horns of the uterus removed under aseptic conditions and placed in two sterile Petri dishes. As much of the work as possible is now done inside a box with a glass top and side doors, similar to that described by Strangeways [1924]. Absolute sterility must be maintained throughout the lengthy processes involved. The embryos are removed from the uterus, decapitated and eviscerated, and, if they are well developed, it is advisable to remove the backbone. The kidneys are removed and placed in a separate dish. The embryonic tissue is chopped as finely as possible, and mixed with Ringer's solution. It is probable that different tissues require Ringer solutions of different composition, but for kidney we have used that described by Pannett and Compton [1924]. The solution is very lightly centrifuged in a hand machine. The upper layer of fluid, which will contain most of the blood cells, is removed, and the minced tissue again mixed with Ringer's solution. It may be necessary again to remove the supernatant liquid layer, but usually hard centrifuging will now leave a faintly opalescent fluid, free from cells. The removal of the blood-cells is most important, as their presence in the culture medium inhibits growth. The cell-free extract thus

obtained nearly always requires further dilution with Ringer's solution, the final strength most suitable for growth being a matter which can only be determined as the result of some experience. A little practice soon enables one to prepare extracts which are remarkably constant in their ammonia content, as the following figures (expressed in mg. ammonia-N per 2 cc. extract) will show:

0.026   0.023   0.026   0.025   0.030   0.022

The kidneys must be freed from all adhering blood, and the number required for each experiment of a series placed in separate dishes, where they are then cut into small pieces. For each experiment we have used 2 cc. of extract, and generally two kidneys. We have taken as much care as possible to use the same amount of tissue throughout any one series of experiments, but owing to the strict aseptic conditions which have to be maintained, it has been found quite impossible to use weighed amounts of tissue. The wet weight of two embryo kidneys from each litter can be determined (*e.g.* 29.5, 44.0, 25.0, 34.0 mg.), and this weight taken as representative of the weight of the remaining kidneys of that litter.

In order that growth may take place, it is necessary for the tissues to have a surface to which they can attach themselves, and for them to be kept moistened and surrounded by the culture medium without being submerged in it. It is thus necessary to use a flask or dish of such a size that the right depth of fluid is obtained when a given quantity of the medium (in this case 2 cc.) is placed in it. In a few of our early experiments we used 100 cc. conical hard-glass flasks with a layer of filter paper on the bottom, but satisfactory growth was not obtained on this, and it was therefore discontinued. Kidney tissue undoubtedly grows best on a glass surface, but if the dish is not absolutely level on the bottom there is danger that a certain amount of tissue will float in the medium, and thus be prevented from growing. We found it almost impossible to obtain dishes or flasks sufficiently even, and on the whole we have found that a very thin layer of cotton-wool has proved quite satisfactory. The pieces of tissue rest on the strands, kept moist by the medium, but in contact with the air. Rubber stoppers or dish covers must be well coated with paraffin wax to prevent evaporation and infection. Incubation at 37° is allowed to proceed for 48 hours. It is generally possible to tell by the naked eye whether or not the culture is sterile after incubation, but as a routine we make smear preparations, which are then stained by Gram's method, and examined with an oil immersion lens. If there should still be any doubt, a drop of the culture medium should be plated out on agar. No analyses have ever been carried out on any but completely sterile cultures.

The culture is filtered into a dry, weighed, hard-glass test-tube, the flask or dish washed with successive small quantities of ammonia-free water, the washings being passed through the filter and added to the filtrate. The weight of the contents of the tube is determined, and aliquot parts are taken for estimation. The filtrate should be kept in the ice-chest till required.

All apparatus for this work must be sterilised, having first been specially cleaned and prepared. In particular, it is important that no trace of alkali should be present on any of the glassware. As a matter of routine we have washed all the glass in sulphuric acid and dichromate, followed by a thorough rinsing in running water, then in distilled water, and finally in ammonia-free water. Each dish, pipette, and cork is separately wrapped in tissue-paper before being sterilised.

For estimation of the ammonia we have used Stanford's method [1923] which we have found to be very accurate until amounts of ammonia-N less than 0.012 mg. are being measured when it is liable to a 10 % error. For urea we have incubated an aliquot part of the filtrate with urease paper [Folin, 1923], and have then estimated the total urea- and ammonia-N, the urea-N being obtained by difference.

#### EXPERIMENTAL.

An elaborate system of controls is necessary:

- A. 2 cc. of medium, without incubation.
- B. 2 cc. of medium, plus the same amount of tissue as that used for the growth flasks (*i.e.* generally two kidneys), without incubation.
- C. 2 cc. of medium, incubated for 48 hours.

A and B are kept at 0° until required.

Thus the total control will be given by:

$$A + (B - A) + (C - A) = B + (C - A),$$

where B will give the amounts of urea and ammonia nitrogen in the medium and tissue before incubation, and (C - A) will give any due to autolysis of the medium during the 48 hours' incubation. Typical figures obtained for these controls are given in Table I.

Table I.

Exp.	Mg. ammonia-N				Mg. urea- + ammonia-N			
	3	6	9	13	3	9	13	16
A	0.026	0.032	0.026	0.030	0.034	0.050	0.045	0.028
B	0.030	0.041	0.032	0.032	0.033	0.057	0.047	0.038
C	0.032	0.040	0.033	0.038	0.042	0.060	0.044	0.029
Total control	0.036	0.049	0.039	0.040	0.041	0.067	0.047	0.039

Figures for A and C are for 2 cc. of extract, figures for B are for 2 cc. of extract + two kidneys.

Each experiment consisted of a series of controls and growing preparations, in all of which the same medium and kidneys taken from embryos of the same litter were employed.

It will be seen that there is an increase of ammonia-N in the medium due to autolysis. No increase of urea was found. The amount of urea present in the kidneys is also extremely small, always within the limits of experimental error, so that sometimes no difference at all was obtained between the total urea- and ammonia-N figures and those for ammonia-N alone, occasionally small minus results even were obtained. It is therefore a matter of doubt as to



whether any urea is present or not in the embryonic kidney. The amount present in the embryonic extract was also very small, less than half that of the ammonia-N. The total amount of ammonia- + urea-N as estimated is of course outside the limits of experimental error, but the difference due to urea-N alone falls within that limit.

In addition to these controls, we devised a further control (D), which we have called the "resting tissue." This consists of the same amount of extract and tissue as the contents of the growth flasks, and like them is incubated for 48 hours. But by using a smaller flask for this resting tissue, we have prevented it from attaching itself to the surface of the glass, and thus also prevented growth from taking place. We have aimed at such a depth of fluid that, although the tissue is freely movable in it, it is only just submerged, and is able to obtain sufficient oxygen to prevent its death. It is obvious from the results of this resting tissue, compared with the total control (Table II), that autolysis has not taken place. We therefore concluded that the tissue was still living, as autolysates of adult mammalian kidneys form both ammonia and urea [McCance, 1924; Luck, 1924, 1]. It was, however, important to make sure of this point; so on one or two occasions at the end of 24 hours' incubation, the flask containing the resting tissue was opened, and part of the medium with some of the pieces of floating tissue was removed to a larger flask, and again sealed. The few pieces remaining behind in the original flask were stained and examined. No sign of growth could be detected. The new flask now contained such an amount of fluid that the remaining pieces of tissue could, if they were still living, attach themselves to the surface of the glass. After a further 24 hours' incubation they were stained and examined, and gave unmistakable evidence of growth, budding having taken place quite freely. This resting tissue having been proved to be living but non-growing tissue serves as a valuable control to the flasks in which growth has taken place. Any difference between ammonia- and urea-N in the growing and resting tissue, both having been subjected to the same conditions and compared with the "total control," can with some confidence be attributed to the actual growth of the tissue. The metabolic activity of the resting tissue seems to be reduced to an absolute minimum consistent with life, and may be described as its resting metabolism.

Table II. *Resting tissue compared with total control.*

	Resting tissue mg. ammonia-N + urea-N	Total control mg. ammonia-N + urea-N
<i>Exp.</i> 13 a	0.063	0.055
13 b	0.050	0.055
13 c	0.039	0.040
19	0.064	0.062

In view of the fact that the resting tissues must be considered to be living for at least the first 24 hours of the experiment we were interested to find a marked difference between these and the growing tissues as regards their activity in producing ammonia and urea. This has already been stated in a

preliminary paper [Holmes and Watchorn, 1927] and can therefore be discussed only very briefly here. In the case of the floating tissue the amounts of ammonia and urea after two days' incubation are exactly the same as the amounts contained in the total control. Examples are given in Table II, to which attention has already been drawn, and they show that the tissue has produced no ammonia or urea.

The growing tissue, on the contrary, produces considerable amounts of ammonia or urea or both together, as may be seen from the figures given in Table III. One might expect that, if this occurred to any extent during the life of the embryo, some sign of it would be found even in the floating tissues, but it must be remembered that the tissue has been allowed to become cold in the interval necessary for the preparation of the medium and the planting of the tissues. Cooling is very effectual in causing cessation of cell division in mammalian cells, and thus, if this production of ammonia and urea is associated with growth, as we believe it to be, it would only be found in cases where the growth had begun again.

In order to compare the results given by the resting and by the growing tissues, it was necessary to be certain that these latter had, in fact, grown. It was not, of course, possible to stain and examine preparations, and afterwards use them for estimation. We have sometimes resorted to the plan of growing special controls for staining, and we have sometimes been able to stain fragments of tissue which remained sticking to the glass after the vessel had been washed out several times for estimation purposes. As a general rule it is quite possible to detect growth with the aid of a binocular dissecting microscope without staining (the vessel merely being placed on black paper). and by this means it is possible to examine preparations in which the ammonia and urea are later to be estimated.

As it happens, in the case of embryo kidney tissue it has been found so consistently that tissues which are not growing form no urea or ammonia, that when these substances increase in amount, it may almost be assumed that growth has occurred. Floating preparations in which a few fragments of tissue have stuck to the glass and grown, show small increases in ammonia and urea. On the other hand, if a "growing" preparation fails to grow (this happened on two occasions, and the failure was eventually recognised as being due to an insufficiency of magnesium in the Ringer's solution), no increase in either of these substances is found.

The results of our experiments with "resting" kidney tissue agree well with those obtained by Warburg [1924] with sliced adult rat kidney tissue. He found that a considerable amount of ammonia appeared as the experiment progressed; but finding that this apparent production of ammonia by the kidney tissue had a very small temperature coefficient, he concluded that he was not dealing with a chemical process, but that the ammonia was merely being washed out of the tissue. This ammonia, which he considered to be existing preformed in the tissue, would be estimated in our tissue control B.

Table III.

Resting or control		Growing tissue		Increase	
Ammonia-N (mg.)	Urea-N (mg.)	Ammonia-N (mg.)	Urea-N (mg.)	Ammonia-N (mg.)	Urea-N (mg.)
0.036	0.011	0.075	0.034	0.039	0.023
0.036	0.011	0.058	0.012	0.022	Nil
0.039	0.029	0.070	0.023	0.031	Nil
0.055	0.022	0.096	0.039	0.041	0.017
0.055	0.022	0.060	0.055	Nil	0.033
0.058	0.015	*0.050	0.033	*Nil	0.018

\* Poor growth.

It will be seen from the examples given in Table III that the amounts of ammonia and urea produced during growth are considerable, and far beyond the limits of the experimental error of this method. It is quite usual for the extra amount of ammonia produced to be equal to 0.1 % or more of the original wet weight. As examples the following experiments may be quoted:

Exp. 18 c. Wet weight 34 mg. Urea increased 0.033 mg.

Exp. 3 a. Wet weight 33 mg. Urea increased 0.023 mg.

Ammonia increased 0.039 mg.

We have no data as yet which might be helpful in showing to what chemical processes we must relate the production of these metabolites. Presumably proteins or similar substances (Carrel and Baker [1926] claim that, in the case of chick tissues growing *in vitro*, proteoses are the most important nutritive constituent of the medium) contained in the embryo extract must be broken down to their constituent amino-acids to be rebuilt into the cells of the growing tissue. Deamination of the amino-acids not required for the synthesis of cell proteins might then occur. If the deamination represents protein combustion for the purpose of obtaining energy for growth, we should be able to prevent this to a considerable extent by adding glucose to the medium, and this we propose to attempt shortly.

Nash and Benedict [1921] showed that during life the kidneys were capable of producing very considerable amounts of ammonia when the glomerular filtrate was acid. The mechanism of this ammonia production is not known. It was attempted at one time to account for it by assuming the presence of urease in the kidney, but no urease could be found when experiments were carried out *in vitro*. In our work we find no evidence for the existence of kidney urease, since the relative and absolute amounts of urea and ammonia present remain unchanged during 48 hours' incubation of our "resting" tissues.

There is, of course, no reason for assuming that the mechanism of this ammonia production is the same as the mechanism of its production by growing tissues. In fact in our growing tissues it seems that it may be urea or ammonia which is produced, or even both together (see Table III), and, according to the evidence of Bollman, Mann and Magath [1924] adult functioning kidney produces no urea. It should be pointed out that in the experiments of Bollman, Mann and Magath the kidneys were plentifully supplied

with glucose, which would tend to prevent urea formation if this originated in protein breakdown. We may further find that a supply of glucose to our growing tissues prevents the formation of urea in them also, or it may be that the metabolism of the growing tissue is in this respect quite distinct from that of the functioning adult tissue.

#### EXPERIMENTS WITH BRAIN TISSUE.

In addition to the experiments just described on kidney tissue, we have carried out the same technique using embryo brain tissue, and while we have not progressed far enough in this part of the work to allow any very definite conclusions to be drawn, one or two points seem to be quite well established and may be worth describing here, though later we hope to be able to deal with brain tissue in more detail.

In the case of brain tissue we have found it more difficult to obtain good growth, and to be certain when growth has occurred. In the experiments with kidney tissue we found it expedient, owing to the small size of the kidneys, to use fairly old embryos, and these gave very good growth. In the case of brain tissue, however, we found it more satisfactory to use young embryos, and even in these one cerebral hemisphere provides plenty of tissue.

The "resting" preparations gave results similar to those obtained with "resting" kidney tissue, in that the ammonia- and urea-N, taken together, showed no increase over the calculated amount in the total control, in fact, slight decreases were observed.

One remarkable fact was noticed, namely, that there was always some suggestion of an increase of ammonia at the expense of urea, that is, a suggestion of urease action. To obtain definite evidence of this, it is necessary to keep the filtrate from the ice-chest tissue control as cold as possible until it is required for estimations. Otherwise the urea in this control will also decrease in amount.

When such precautions are taken, results similar to those given in Table IV are obtained.

Table IV.

				Resting tissue (mg.)	Total control (mg.)
Ammonia-N	...	...	...	0.053	0.033
Urea-N	...	...	...	0.015	0.038
Ammonia-N + urea-N	...	...	...	0.068	0.071

So far the occurrence of urease in the tissues of higher animals has only been reported for the gastric mucosa of certain species [Luck, 1924, 2], but we have no reason so far to doubt that it is actually present in rat brain tissue, although probably only in small amounts. Adult rat brain tissue has been found to convert a little added urea to ammonia, but only a small number of experiments have been carried out. Rabbit brain tissue gave definitely negative results, showing no sign at all of the presence of urease. We have injected several rats subcutaneously and intraperitoneally with urea. With 1 g.

of urea the rats showed rigidity of the tail, twitchings and finally drowsiness, but with more urea (about 2 g. given at intervals) severe convulsions occurred about three-quarters of an hour later. So far as we know this effect has not been described for other animals known to contain no urease, as for example the rabbit; and while we cannot yet state that the effect we obtained in the case of the rat was due to the conversion of urea into ammonia, the possibility must be considered and further investigated.

In the case of the growing preparations we have obtained only a few satisfactory results, and these are most unlike those obtained with growing kidney tissue. When good growth has taken place we have usually found a considerable fall in the ammonia- and urea-N taken together. This suggests that some synthesis of nitrogenous substances at the expense of ammonia and urea must take place when the tissue is actively growing. More experimental data will have to be obtained, however, before this view can be expressed with certainty, and this work is now in progress.

#### SUMMARY.

1. The technique of tissue culture has been employed to study the nitrogenous metabolism of growing tissues.
2. When growing, embryonic rat kidney tissue produces considerable amounts of ammonia and urea.
3. "Resting" embryonic kidney tissue, incubated under exactly the same conditions as the growing tissue, produces neither ammonia nor urea.
4. Some preliminary experiments with brain tissue are described and the results contrasted with those obtained with kidney tissue. The probable existence of urease in the rat brain is discussed.

In conclusion we wish to express our indebtedness to Mr R. A. McCance, who was working with one of us (B. E. H.) for more than a year at this investigation when it was first undertaken, and to whom we owe many points of technique and many suggestions.

We wish also to thank Sir F. G. Hopkins for his kind interest in this work.

#### REFERENCES.

- Bollman, Mann and Magath (1924). *Amer. J. Physiol.* **69**, 371.  
Carrel and Baker (1926). *Proc. Soc. Exp. Biol. Med.* **23**, 627.  
Folin (1923). *Lab. Manual of Biol. Chem.* p. 230.  
Holmes and Watchorn (1927). *Compt. Rend. Soc. Biol.* **96**, 691.  
Luck (1924, 1). *Biochem. J.* **18**, 814.  
—— (1924, 2). *Biochem. J.* **18**, 825.  
McCance (1924). *Biochem. J.* **18**, 486.  
Nash and Benedict (1921). *J. Biol. Chem.* **48**, 463.  
Pannett and Compton (1924). *Lancet*, i, 381.  
Stanford (1923). *Biochem. J.* **17**, 847.  
Strangeways (1924). *Technique of Tissue Culture*, p. 1.  
Warburg (1924). *Bioch. Z.* **152**, 309.

# XLVII. THE CATALYTIC ACTION OF TRACES OF IRON AND COPPER ON THE ANAEROBIC OXIDATION OF SULPHYDRYL COMPOUNDS.

BY DOUGLAS CREESE HARRISON.

*From the Biochemical Laboratory, Cambridge University, and  
the Department of Pharmacology, Sheffield University.*

*(Received February 20th, 1927.)*

IN a previous publication by the author [1924], it was shown that the rate of oxidation of glutathione and cysteine by the oxygen of the air was greatly reduced by the addition of traces of hydrogen cyanide. It was shown also that the rate of oxidation of glutathione and cysteine could be reduced to a similar low value by very careful purification in quartz vessels, taking all possible precautions to exclude iron; and further, that the rate of oxidation of the purified compounds could be accelerated by the addition of minute traces of iron. Evidence was given in support of Warburg's view that the inhibition by cyanide is due to the formation of a non-catalytic complex with traces of catalytic metals ordinarily present as impurities in the sulphydryl compounds.

During the course of these experiments, I found that not only the aerobic but also the anaerobic oxidation of sulphydryl compounds was greatly inhibited by small concentrations of hydrogen cyanide. In these anaerobic oxidations methylene blue acted as the hydrogen acceptor in place of oxygen. It seemed possible that, as in the case of the aerobic oxidation, the cyanide might be acting on traces of catalytic metal impurities. If this were true, these metals would be expected to catalyse not only the aerobic but also the anaerobic oxidation of sulphydryl compounds, and could not be regarded as acting as carriers or activators of oxygen as pictured by Warburg [1924]. It was therefore desirable to obtain samples of sulphydryl compounds and methylene blue as free as possible from traces of catalytic metals, and then to show that the rate of anaerobic oxidation could be accelerated by the addition of traces of iron to the purified compounds.

The present paper contains the results of these experiments. Most of these results were obtained in 1924, and a brief reference to them was made in a paper by Thurlow [1925]. In 1926, a paper appeared by Toda [1926], containing experiments similar to some of those which I am about to describe.

Both his and my own experiments yielded a similar result, and he draws the same conclusion as was indicated in the reference to my experiments by Thurlow [1925].

*Inhibition by hydrogen cyanide.*

The following experiments show the great inhibition produced by traces of hydrogen cyanide on the reduction of methylene blue by cysteine, glutathione and thioglycollic acid. In this group of experiments, the vacuum tubes and technique described by Thunberg were used. After twice evacuating and filling with nitrogen, the tubes were again evacuated and immersed in a water-bath, the latter being mechanically stirred and maintained at 37°.

The vacuum tubes each contained 0.3 cc. of methylene blue (1 part in 5000 of water) and a solution of the particular sulphydryl compound neutralised with dilute sodium hydroxide. 0.1 cc. of potassium cyanide solution (previously neutralised with HCl) was added to one tube and an equal volume of water to the other tube. The total volume of solution in each tube was made up to 2.0 cc. with phosphate buffer.

Table I.

Sulphydryl compound	$p_{H}$	Time in min. for decoloration of Mb
Cysteine (0.3 mg.) ... ..	7.0	Without cyanide 12 min.
"	"	+ $M/4000$ HCN 42
"	7.0	Without cyanide 12
"	"	+ $M/8000$ HCN 27
"	7.4	Without cyanide 12
"	"	+ $M/2000$ HCN 45
"	7.6	Without cyanide 10
"	"	+ $M/2000$ HCN 60
Glutathione (1.2 mg.) ... ..	7.0	Without cyanide 35
"	"	+ $M/2000$ HCN 110
Thioglycollic acid (1.2 mg.)	7.4	Without cyanide 95
"	"	+ $M/4000$ HCN 240

From these experiments it is evident that the addition of very small quantities of cyanide produces a marked inhibition in the rate of reduction of methylene blue by sulphydryl compounds.

On adding iron to solutions containing cyanide, it was found that the inhibition produced by the latter was considerably reduced. On adding iron to the solutions of sulphydryl compounds in the absence of cyanide, the rate of reduction was increased, though this increase was not very marked, owing to the relatively large amount of iron already present in the solutions.

*Preparation of iron-free methylene blue.*

The method used for preparing iron-free methylene blue was similar to that previously employed by the author in purifying cysteine [Harrison, 1924]. It depends, firstly, on the precipitation of the iron by hydrogen sulphide in presence of baryta, and, secondly, on crystallisation of the leuco-compound from pure alcohol. In view of the fact that cysteine forms a compound with

iron while methylene blue shows no such tendency, it seemed likely that the method would give even better results than with cysteine.

Two grams of crystalline methylene blue (the free base) were added to 150 cc. of water in a Pyrex conical flask which had been previously boiled out with pure concentrated hydrochloric acid. 20 g. of finely powdered crystalline baryta were then added and, after shaking, a rapid stream of well-washed hydrogen sulphide was passed for half an hour. The methylene blue was reduced to the leuco-compound which, together with the iron, was thrown down as a greyish precipitate. This was allowed to stand for 24 hours in a closed vessel, and then shaken with 100 cc. of moist ether which had been previously distilled from quartz vessels and saturated with hydrogen sulphide. The ether dissolves most of the leuco-compound, leaving the iron behind as a greenish basic sulphide. After standing for 2 hours the greater part of the ether layer was carefully poured into a quartz flask, shaken with a few cc. of pure water to remove any traces of barium, and poured off into an acid-extracted filter paper resting in the neck of a quartz flask. After filtering, the mouth of the flask was covered with a filter paper and the ether was distilled off, leaving the pale yellow leuco-methylene blue. This was dissolved in the minimum quantity of hot alcohol (distilled from quartz and containing a little dissolved hydrogen sulphide), half the volume of pure water was added and the solution was cooled under the tap for a short time.

Crystalline leuco-methylene blue separated out in fine needles and was filtered in a quartz Gooch crucible lined with an acid-extracted filter paper, and washed several times with 70 % alcohol (which had been distilled in quartz). If care be taken in the preceding operation to avoid undue exposure to the air, the leuco-methylene blue is obtained quite free from the oxidised form. After drying on the pump for a few minutes, the leuco-compound started to oxidise rapidly. The drying was completed in a vacuum desiccator over sulphuric acid.

The yield obtained under these conditions is small, but may be increased, though at the possible sacrifice of purity, by crystallising from more dilute alcohol.

#### *Purification of sulphhydryl compounds.*

Cysteine hydrochloride was rendered free from traces of iron by the method employed in experiments on the aerobic oxidation of cysteine by Sakuma [1923] and Harrison [1924].

Thioglycollic acid was purified from traces of metallic impurities by distillation in hydrogen at 16 mm. Hg pressure, using quartz vessels.

A large number of experiments was then carried out on the rate of reduction of the pure methylene blue by the pure sulphhydryl compounds. Preliminary experiments were carried out in quartz vacuum tubes, but it was found difficult to obtain good agreement between duplicates, possibly owing to the presence of oxygen adsorbed on the walls of the quartz tubes. It was



found, however, that by subjecting glass vacuum tubes to a thorough cleaning with acids before each experiment, contamination of the solutions by traces of catalytic metals did not occur, and that the difficulties associated with the use of quartz vacuum tubes could be obviated.

Owing to variations in the minute quantities of metals remaining in different samples of the purified compounds and to the difficulty of ensuring that absolutely no metallic impurities are introduced during the making up of the solutions, the reduction times observed in different experiments employing the same quantities of sulphhydryl compound and methylene blue varied somewhat. Another possible source of this variation may be the differences in the intensity of illumination of the water-bath on different days. Preliminary experiments appear to indicate that sunlight accelerates the rate of reduction of methylene blue by cysteine and thioglycollic acid, and Dixon and Tunnicliffe [1923] observed a similar effect in the case of glutathione. Nevertheless, these facts do not in any way affect the conclusions drawn in this paper on the relative rates of anaerobic oxidation of sulphhydryl compounds in the presence and absence of catalytic metals, for in every experiment duplicate tubes were employed and these duplicates almost invariably showed good agreement. In the few cases where good agreement was not obtained, the results were discarded and the experiments repeated.

Before each experiment, the glass tubes were treated with small quantities of a mixture of concentrated nitric and sulphuric acids to which a few drops of alcohol were cautiously added. After repeating this treatment, the tubes were boiled out with pure concentrated hydrochloric acid and allowed to stand filled with concentrated hydrochloric acid. Finally the tubes were well rinsed out with quartz-distilled water. After this treatment the tubes were found to be perfectly satisfactory and to give good duplicates. The lower part of the ground glass stoppers was left ungreased to avoid possible contamination of the solution from the tap grease. After dissolving the pure sulphhydryl compound in quartz-distilled water, the solutions were brought to the required  $p_H$  by the addition of dilute ammonium hydroxide previously distilled in quartz vessels. The alkali was added from a quartz burette, the volume required being determined by separate titration. No buffer was used in the experiments with the purified compounds. The tubes were evacuated with the water-pump and filled with nitrogen (washed with alkaline pyrogallol). This was repeated, and finally the tubes were evacuated and completely immersed in the water-bath, the side tubes being first filled with water to reduce the chance of leakage. The  $p_H$  was checked at the end of each experiment by means of Clark and Lubs' standard indicators. A few drops of the indicator previously boiled to expel any dissolved air were introduced into the side tube of each reduction tube and allowed to mix with the solution inside the tube by partially turning the stopper, care being taken to prevent the entry of any air. In all experiments described in this paper, 0.3 cc. of purified methylene blue (1 part dissolved in 5000 parts of quartz-distilled water) was used in each tube. The total volume

of solution in each tube was always made up to 2.0 cc. In all experiments employing iron, a freshly prepared solution of ferric chloride was used. The copper used was added as copper sulphate solution. The results quoted in the following tables are those of typical experiments chosen from a large number. Each experiment was carried out in duplicate.

Table II shows the catalytic effect of traces of iron on the anaerobic oxidation of purified cysteine at 37°.

Table II.

Each tube contained 0.4 mg. of purified cysteine hydrochloride.  
Iron added as ferric chloride. Temp. 37°.

No.	p <sub>H</sub>	Iron added (mg.)	Time in min. for decoloration of Mb
1	7.3	—	18 min.
	7.3	0.01	9
2	7.0	—	53
	7.0	0.001	24
3	7.7	—	23
	7.7	0.001	15

It was found that by carrying out the experiments at 25° instead of at 37° the rate of decoloration of methylene blue was slower, and that consequently differences in reduction time could be measured more easily. All the experiments subsequently to be described were therefore carried out at 25°. Table III shows the catalytic effect of traces of iron on the anaerobic oxidation of purified cysteine at 25°.

Table III.

Iron added as FeCl<sub>3</sub>. Temp. 25°.

No.	Cysteine-HCl mg.	p <sub>H</sub>	Iron added mg.	Reduction time of Mb in min.
1	0.4	8.5	—	90
			0.001	55
2	0.5	8.4	—	39
			0.001	18
3	0.8	7.8	—	98
			0.001	33
4	0.8	7.6	—	50
			0.001	14
5	0.8	5.6	—	73
	"	"	0.001	57

Table IV.

Each tube contained 0.8 mg. of purified cysteine hydrochloride.  
Copper added as CuSO<sub>4</sub>. Temp. 25°.

No.	p <sub>H</sub>	Cu added mg.	Reduction time of Mb in min.	Reduction time of Mb with HCN
1	7.6	—	50	—
	"	0.0001	9	—
	"	0.001	< 1	—
2	7.4	—	66	+ M/1000 HCN 65 min.
	"	0.0001	8	—
	"	0.0002	6	—
3	7.6	—	60	+ M/500 HCN 72 min.
	"	0.00001	26	—
	"	0.00005	9	—

It is evident from Tables II and III that iron acts as a powerful catalyst in the anaerobic oxidation of cysteine, both on the acid and alkaline side of neutrality.

Table IV shows the very powerful catalytic effect of minute traces of copper on the anaerobic oxidation of purified cysteine.

For purposes of comparison, it may be mentioned that a sample of ordinary unpurified cysteine hydrochloride (0.8 mg.) in  $p_H$  7.6 phosphate buffer under the same experimental conditions as in Table IV reduced the methylene blue in 10 minutes in the absence of cyanide, while in presence of  $M/1000$  HCN, it reduced in 70 minutes; on the other hand it will be seen from *Exp.* 3 in Table IV that under similar conditions 0.8 mg. of *purified* cysteine hydrochloride reduced the methylene blue in 60 minutes without HCN, and in 72 minutes after the addition of  $M/500$  HCN. From Table IV there is seen to be very little, if any, inhibition produced by HCN on the rate of oxidation of the purified cysteine. From the results in Table IV it is evident that a quantity of copper so minute as five millionths of a milligram per cc. produces a considerable acceleration in the anaerobic oxidation of cysteine. By comparison with Table III, it will be seen that iron is considerably less active as a catalyst than copper. Nevertheless, five ten-thousandths of a milligram of iron per cc. was found to produce a considerable acceleration.

A number of experiments were then carried out using purified thioglycollic acid instead of cysteine and it was found that in marked contrast to cysteine, the rate of reduction of methylene blue by purified thioglycollic acid was not appreciably increased by the addition of iron in quantities of the same order as those employed in the experiments with cysteine. On the other hand, a trace of copper was found to act as a very powerful catalyst in the anaerobic oxidation of purified thioglycollic acid. This is shown in Table V.

Table V.

No.	Copper added as $\text{CuSO}_4$ . Temp. $25^\circ$ .		Cu added (mg.)	Reduction time of Mb in min.
	Thioglycollic acid (mg.)	$p_H$		
1	1.2	7.4	—	137
	"	"	0.001	< $\frac{1}{2}$
	"	"	0.0005	10
2	1.6	7.6	—	35
	"	"	0.001	3
3	1.2	7.9	—	155
	"	"	0.001	6

The addition of  $M/1000$  HCN to the purified thioglycollic acid was found to produce practically no inhibition, though the oxidation of a solution of the pure thioglycollic acid to which a minute trace (0.0005 mg.) of copper had been added was markedly inhibited by  $M/1000$  HCN.

In investigating the effect of iron on the anaerobic oxidation of purified thioglycollic acid, a slight acceleration appeared to be induced by relatively large quantities of iron, but in view of the extremely powerful effect of copper on this oxidation, it seems possible that this effect observed with larger con-

centrations of iron may be due to traces of copper (or possibly some other catalytic metal) present in the iron solutions. Owing to the non-sensitivity of thioglycollic acid to iron, and to its ready purification by a single distillation in quartz, it is easy to obtain solutions of this compound the rate of oxidation of which is not appreciably inhibited by the addition of HCN. On the other hand, such solutions of cysteine are more difficult to obtain.

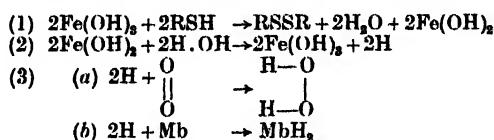
There seems little reason to believe that the action of catalytic metals on the anaerobic oxidation of pure glutathione would be essentially different from their action on pure cysteine, for in the experiments on the aerobic oxidation of purified glutathione, it was shown that traces of iron produced a similar catalytic effect on the purified glutathione to that produced on purified cysteine [Harrison, 1924]. Further, it is shown in the present paper that the anaerobic oxidation of glutathione is inhibited by traces of cyanide in a similar way to that of cysteine and thioglycollic acid. It seems highly probable, therefore, that the anaerobic oxidation of pure glutathione is catalysed by traces of metals in a similar way to that of cysteine.

#### DISCUSSION.

In a recent paper by Moureu, Dufraisse and Badoche [1926], it is suggested that the inhibitions caused by hydrogen cyanide are not necessarily to be explained by the inactivation by cyanide of traces of catalytic impurities, but that the facts can equally well be explained by the general "antioxygen" theory of Moureu and Dufraisse [1926]. According to this theory, the substance which is oxidising and the substance which is causing inhibition both take up oxygen to form compounds of the peroxide type. The interaction and mutual decomposition of the two peroxides then takes place. This leads to the regeneration of the original unoxidised substances, and thus oxidation is prevented. These authors suggest that the inhibition caused by hydrogen cyanide in the oxidation of sulphydryl compounds can be explained in the above manner, but that such is not the case is evident from the following considerations. Firstly, the more carefully the sulphydryl compounds are purified from traces of catalytic metals, the less becomes the inhibition produced by cyanide [Harrison, 1924]. Secondly, pyrophosphates have been shown to inhibit the oxidation of sulphydryl compounds in a similar way to cyanides, and in this case the formation of a transitory peroxide of the inhibitor (as required by the antioxygen theory) can hardly be taking place. Finally, the experiments described in the present paper show that the inhibition by cyanide also occurs in the *anaerobic* oxidation of sulphydryl compounds, a reaction in which formation of peroxides of the substance oxidising or of the inhibitor can scarcely be regarded as possible.

From the results of the experiments given so far in this paper, it is evident that the anaerobic oxidation of the sulphydryl group is accelerated by traces of iron in a similar way to its aerobic oxidation. In the experiments on the anaerobic oxidation, methylene blue replaces oxygen as the hydrogen acceptor.

Since iron produces a similar catalytic effect on both the aerobic and the anaerobic oxidations of the sulphhydryl group, it would appear that the iron cannot be acting as an activator of oxygen as suggested in Warburg's theory. It would seem rather that the experimental facts can be satisfactorily explained by assuming that in both the aerobic and anaerobic oxidation of the sulphhydryl compounds, the iron (or other catalytic metal) is acting as an intermediary catalyst, becoming alternately oxidised and reduced. Ferric iron is reduced by the sulphhydryl compound; the ferrous compound thus formed then reduces the methylene blue to methylene white in the anaerobic experiments, or oxygen to hydrogen peroxide in the aerobic experiments. The resulting ferric compound is then again reduced by a further quantity of the sulphhydryl compound, so bringing about the oxidation of the latter. The essentials of the process may be expressed as follows:



Reaction (2) will proceed in a forward direction in the presence of a hydrogen acceptor such as oxygen or methylene blue, so long as the reduction potential of the system expressed by  $\text{Fe}^{\cdot\cdot}/\text{Fe}^{\cdot\cdot\cdot}$  is sufficiently high to reduce the hydrogen acceptor.

This condition is ensured by the re-reduction of the ferric iron by the excess of sulphhydryl compound in the solution, the reduction potential of the system  $\text{Fe}^{\cdot\cdot}/\text{Fe}^{\cdot\cdot\cdot}$  thus being maintained. Dixon [1927] has recently described experiments which indicate that the reduction potential of the sulphhydryl system is considerably higher than that indicated by previous work. The sulphhydryl compounds will tend to maintain the reduction potential of the ferrous-ferric system at a value well above that of the system  $\text{MbH}_2/\text{Mb}$ , and the reduction of the methylene blue will proceed virtually to completion. In such a mechanism, the velocity of oxidation of the sulphhydryl compound will be determined by the slower of the two reactions, namely, the reduction of the ferric compound by the sulphhydryl group, and the subsequent reduction of the hydrogen acceptor by the resulting ferrous compound. A positive catalysis by iron will occur provided that the slower of these two reactions is more rapid than the direct reduction (without the intervention of iron) of the hydrogen acceptor by the sulphhydryl compound.

Such a conception of iron as an intermediary catalyst does not appear to necessitate the assumption of an activation of oxygen by iron as pictured by Warburg. It would appear rather that molecular oxygen is acting as an acceptor for hydrogen formed in reaction (2). It is probable that the catalysis proceeds by the alternate oxidation and reduction of an iron-sulphhydryl complex rather than by the free inorganic iron indicated in reactions (1) and (2). There is abundant evidence for the existence of this complex. The

assumption of its formation, while it does not modify in any essential the theory outlined above, will explain the non-precipitation of ferric hydroxide from the reacting system during the catalysis in alkaline solution. There seems no reason for assuming an activation of oxygen by iron in the aerobic system any more than we assume an activation of methylene blue by iron in the anaerobic system. The experimental results given in the present paper combined with previous work on the subject indicate that there is no essential difference between the catalysis in the aerobic and in the anaerobic systems, and the mechanism outlined above would explain the catalysis in the two systems on exactly similar lines. A somewhat similar explanation of the catalysis by iron of the oxidation of hexoses in phosphate solution has recently been suggested by Blix [1927].

An alternative possible explanation of the catalysis of the oxidation of sulphydryl compounds is one similar to that suggested by Toda [1926], namely, the activation of the hydrogen of the sulphydryl group by iron. In the formation of the complex between iron and the sulphydryl compounds, the hydrogen of the sulphydryl group is assumed to be activated, and this hydrogen is then readily taken up by methylene blue or oxygen. It would seem, however, that the theory of the alternate oxidation and reduction of the catalytic metal is the more attractive explanation of the facts for several reasons. Firstly, it does not involve the assumption (for which as yet there is no direct evidence) of an activation by iron of the hydrogen of the sulphydryl group. Secondly, there is definite evidence that an alternate oxidation and reduction of iron takes place during the catalytic oxidation of the sulphydryl group. The change of ferrous to ferric iron and *vice versa* can actually be observed by the appearance and disappearance of the violet colour of the ferric-cysteine complex in the alkaline oxidising solution [Harris, 1922], and it was shown by Harrison [1924] that a similar colour change occurs also in glutathione solutions oxidising in the presence of iron. Further, it appears significant in this connection that the metals which were found by Mathews and Walker [1909] to catalyse the oxidation of cysteine are all capable of existing in at least two well-defined different states of valency. The evidence given in favour of the oxidation-reduction theory of catalysis by iron does not, however, exclude the possibility that the direct activation of the hydrogen of the sulphydryl group by iron may also be playing a part. The two mechanisms may be going on side by side.

The experiments described in the present paper, while showing the enormous catalytic effect of certain metals, at the same time appear to indicate that the reduction of methylene blue by sulphydryl compounds can take place with a not inconsiderable velocity, even in the absence of catalytic metals. In the purest solutions of methylene blue and sulphydryl compounds obtained, it was found that the addition of cyanide produced no appreciable inhibition in the rate of anaerobic oxidation. Any traces of catalytic metals remaining in the purified solutions would presumably be inactivated by the

cyanide, and yet, even in the presence of cyanide, the velocity of the reaction was found to be practically unaltered. It is of interest to compare this result with the results of experiments obtained by the author [Harrison, 1924] in the case of the aerobic oxidation of glutathione and cysteine. It was found in these aerobic experiments that cyanide did indeed produce an inhibition in the rate of oxidation of the purified compounds, though, even in the presence of excess of cyanide, an appreciable oxidation of the sulphydryl compounds still occurred. It seems not unlikely, therefore, that the sulphydryl group can oxidise to some extent, even in the absence of catalytic metals, and that these metals act by accelerating a reaction which is already taking place at a measurable rate.

#### OXIDATION OF IRON-FREE METHYLENE WHITE.

According to Warburg, molecular oxygen can under no circumstances act as an acceptor for hydrogen; the molecular oxygen must first be activated by combination with iron. If this view be correct, it follows that the aerobic oxidation of any substance by loss of hydrogen cannot take place in the complete absence of iron. However, during the preparation of iron-free methylene blue (as described above), it was interesting to find that the iron-free leuco-compound oxidised rapidly on exposure to the air. Here was an example of a substance containing two labile hydrogen atoms which could be taken up by the molecular oxygen of the air, even in the absence of iron. The oxidation was evidently not brought about by traces of iron introduced from the air during the drying of the leuco-compound on the pump, for small quantities of leuco-compound which remained behind in the quartz flask were similarly oxidised.

Further evidence for the ability of molecular oxygen to act as a hydrogen acceptor for the labile hydrogen of leuco-methylene blue is afforded by the following experiments.

To each of two vacuum tubes were added 0.1 cc. of cysteine solution (containing 0.3 mg. of free cysteine), 0.3 cc. of 1/5000 methylene blue and 1.5 cc. of  $p_H$  7.6 phosphate buffer. 0.1 cc. of  $M/20$  KCN (previously neutralised with HCl) was then added to one tube and an equal volume of water to the other tube. The tubes were then evacuated and immersed in a water-bath at  $37^\circ$  until the methylene blue was completely decolorised in both tubes. The tubes were then opened to the air and shaken. It was found that the solutions in both tubes became slightly blue almost immediately, and the rate of progressive deepening was the same in both tubes. The tube to which cyanide had been added smelled strongly of HCN at the end of the experiment. In another experiment, 10 cc. of a 1/5000 solution of iron-free methylene blue in pure water was reduced by passing a current of pure hydrogen sulphide (prepared from  $Sb_2S_3$ ) through the solution for half an hour. The solution was then divided into two equal parts in two quartz test tubes. To one tube was added 0.1 cc. of  $M/20$  KCN (carefully neutralised with HCl) and to the

other was added an equal volume of pure water. The tubes were then evacuated on the pump to remove excess of hydrogen sulphide, and, on opening to the air and shaking, the rate of oxidation of the leuco-methylene blue was again observed to be the same in both tubes. Traces of iron impurities in the solution would have been inactivated by the hydrogen cyanide, and since the rate of oxidation was the same, either in the presence or in the absence of cyanide, it is evident that iron is not necessary for the oxidation of leuco-methylene blue, but that the latter can give up hydrogen directly to unactivated molecular oxygen.

This fact is of importance in considering the validity of Warburg's theory. Warburg states that molecular oxygen is entirely unable to act as an acceptor for hydrogen unless first activated by iron. Nevertheless, the above experiment proves that molecular oxygen is capable of combining with the hydrogen of leuco-methylene blue, even in the complete absence of iron. It would appear, therefore, that in certain oxidations at any rate, molecular oxygen *can* function directly as a hydrogen acceptor. It is true that the oxidation of leuco-methylene blue is not actually a biological reaction, but there seems no reason why it should be regarded as essentially different in mechanism from the oxidation of a biological system. As shown by Dixon and Thurlow [1925], the aerobic oxidation of hypoxanthine in the presence of the xanthine oxidase is also unaffected by the addition of cyanide. It would therefore seem that both in the oxidation of leuco-methylene blue and in the oxidation of hypoxanthine in the presence of xanthine oxidase, labile hydrogen is taken up by molecular oxygen without the intervention of iron. Warburg [1924], however, refused to concede that the oxidation of hypoxanthine in the presence of xanthine oxidase is a tissue respiration system. It is difficult to understand why this reaction should not be classed as a part of tissue respiration, since the oxidation of hypoxanthine is known to occur in the tissues.

#### SUMMARY AND CONCLUSIONS.

Briefly, the chief conclusions that may be drawn from this paper are as follows. The fact that hydrogen cyanide inhibits not only the aerobic but also the anaerobic oxidation of sulphydryl compounds indicates that it is not inhibiting an oxygen activator. Such a fact means that inhibition by cyanide in an oxidising system does not justify the assumption that oxygen activation is a necessary process in that system. The observation that the addition of very small traces of certain metals to purified sulphydryl compounds accelerates not only the aerobic but also the anaerobic oxidation of the sulphydryl group affords further indication that the catalytic action of these metals is not that of an oxygen activator. It is suggested that this catalysis of the oxidation of the sulphydryl group by metals is effected by the alternate oxidation and reduction of the catalytic metal by means of which hydrogen is made available for acceptance by molecular oxygen or by methylene blue. Such an explanation makes comparable the mechanism involved in the catalysis of both the



aerobic and the anaerobic oxidation of the sulphhydryl compound. These experiments indicate the important part played by extremely minute traces of iron and other metals in biological oxidations involving the sulphhydryl group. Copper, in the concentrations shown to be effective, may well be regarded as a physiological factor, since small quantities of copper have been shown to be present in many living tissues. Another possible function of small quantities of iron has been indicated by Harrison and Thurlow [1926] in whose experiments it was shown that in the presence of the xanthine oxidase system to which ferrous iron had been added, the oxidation of certain physiological substances such as lactic acid and  $\beta$ -hydroxybutyric acid could be brought about. In this system, the iron appears to be acting as a peroxidase rather than by the mechanism considered above.

The experiments in the present paper indicate that the reduction of methylene blue by sulphhydryl compounds may take place to an appreciable extent even in the complete absence of catalytic metals. A similar result was obtained in an earlier paper in the aerobic oxidation of sulphhydryl compounds. Further evidence against the necessity for oxygen activation in this system is thus afforded. The fact that iron-free leuco-methylene blue has been shown to be strictly autoxidisable affords another example of the direct acceptance of hydrogen by molecular oxygen.

The author wishes to express his sincere thanks to Sir F. G. Hopkins and to Professor E. Mellanby for their interest and encouragement.

He also wishes to express his indebtedness for a Keddey Fletcher-Warr Studentship, University of London, held during the course of this work.

#### REFERENCES.

- Blix (1927). *Skand. Arch. Physiol.* **50**, 8.  
Dixon (1927). *Proc. Roy. Soc. Lond. B*, **101**, 57.  
Dixon and Thurlow (1925). *Biochem. J.* **19**, 672.  
Dixon and Tunnicliffe (1923). *Proc. Roy. Soc. Lond. B*, **94**, 281.  
Harris (1922). *Biochem. J.* **16**, 739.  
Harrison (1924). *Biochem. J.* **18**, 1009.  
Harrison and Thurlow (1920). *Biochem. J.* **20**, 217.  
Mathews and Walker (1909). *J. Biol. Chem.* **6**, 299.  
Moureu and Dufraisse (1926). *Chem. Rev.* **3**, 113.  
Moureu, Dufraisse and Badoche (1926). *Compt. Rend. Acad. Sci.* **183**, 685.  
Sakuma (1923). *Biochem. Z.* **142**, 68.  
Thurlow (1925). *Biochem. J.* **19**, 186.  
Toda (1926). *Biochem. Z.* **172**, 34.  
Warburg (1924). *Biochem. Z.* **152**, 479.

## XLVIII. NOTE ON AN EFFICIENT GAS SCRUBBER.

By HERBERT WILLIAM SOUTHGATE.

*From the Pathological Laboratory, Croydon General Hospital.*

*(Received February 28th, 1927.)*

THE scrubber described and illustrated below is particularly suitable for the analysis of a volatile substance which is based on volatilising the compound and trapping it in a suitable liquid. The author has used the scrubber with advantage in the method of estimating small amounts of alcohol in body fluids as described by Cannan and Sulzer, and also in the estimation of urea in blood by Van Slyke's method. Doubtless it could be used for other similar methods.

The efficiency of the scrubber depends upon the incorporation of one of the well-known glass filter plates made by the famous Schott firm of Jena. All the gas or mixture of gases and vapours has to pass through the pores of the plate and must thus emerge as tiny bubbles (see Fig. 1). Not only is it necessary to get efficient contact between the trapping liquid and the gas mixture, but there must also be an efficient circulation of the former through the inside of the bubbler above the plate. The ideal method of effecting this is to have a small tube through the centre of the porous plate coming up from the bottom of the bubbler. Messrs Schott kindly made some of these to the writer's design some eighteen months ago. One of them is illustrated in Fig. 1, which shows not only the bubbler but also the holder and a ground-on glass container for the trapping liquid. Two of these containers are ground to the same holder and so can be used alternatively to save time. The mixture of gases is drawn through the apparatus in the direction indicated by the arrows. The upward motion of the bubbles above the porous glass plate results in liquid being sucked up through the small centre tube which pierces the plate.

It will be noted that the upper end of the bubbler tube above the plate is just turned down again. This stops splashing and gives another washing (were any necessary) if the level of this turned down end just touches the liquid; this can easily be adjusted if the same volume of a standard liquid is used in the container each time. The advantages of this small piece of apparatus, as illustrated, may be summed up as follows.

1. Thoroughly efficient scrubbing.
2. Thorough circulation of the trapping liquid.
3. Prevention of splashing of the latter up and through the exit tube.
4. It is made entirely of glass. There is thus no possibility of the liquid coming into contact with oxidisable material such as rubber.

5. It can be easily washed out at the end of an experiment, the plate itself and the inlet tube being readily flushed out by connecting the end *A* to a distilled water supply giving a pound or two of pressure.

6. Its relative cheapness—the whole apparatus<sup>1</sup>, including an extra container and the outside vaporising tube (which is not shown in the illustration) costing about 45s.

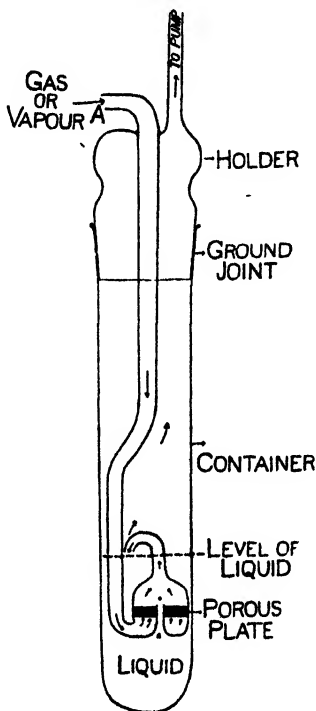


Fig. 1.

<sup>1</sup> Made for me by the Scientific Glass Blowing Company of 12 Wright Street, Oxford Road, Manchester, who keep a stock of these Jena bubblers made to my design.

# XLIX. DECOMPOSITION OF HEXOSEPHOSPHATES BY *B. COLI COMMUNIS*, ESCHERICH.

By RODGER J. MANNING.

*From the Biochemical Department, Lister Institute, London.*

*(Received February 25th, 1927.)*

BOTH hexosemonophosphoric and hexosediphosphoric acids are decomposed by *B. coli communis*, Esch. with production of acid and gas. The following experiments were made to ascertain whether the products of decomposition of these acids differed in any marked way from those formed by the action of the same organism on glucose and fructose. The barium salts of the acids were prepared by the method described by Robison [1922].

On account of the toxicity of the barium salts, as well as of the slight solubility of the barium hexosediphosphate, the sodium salts were formed and used in the experiments. These were decomposed by *B. coli communis* in atmospheres of oxygen and of nitrogen, respectively, at 37° for periods of seven to ten days and the resultant liquids then analysed. The products of decomposition were found to be carbon dioxide, alcohol, formic, acetic, lactic and succinic acids, whether the decomposition took place in presence or absence of oxygen<sup>1</sup>, this being in agreement with the observations of Grey, when decomposing glucose by *B. coli communis* [1919, 1920; Grey and Young, 1921].

The more rapid the bubbling of oxygen through the bacterial digest the greater was the amount of the sugar decomposed completely to carbon dioxide. In the anaerobic digest the amounts of alcohol and of all the acids were markedly greater than in the aerobic, the carbon dioxide being correspondingly less.

## *Purification of barium hexosediphosphate.*

256 g. of crude barium hexosediphosphate were carefully triturated with 2560 cc. water and filtered. The residue was again triturated twice in succession very carefully with 400 cc. water, filtered and the combined filtrates were used for the preparation of the barium hexosemonophosphate. The residue containing insoluble inorganic phosphates and barium hexosediphosphate was now ground up carefully over a period of two hours with 100 parts of water and from this solution by twice precipitating with lead acetate as described by Robison [1922] the purified barium hexosediphosphate used in the experiments was finally obtained.

<sup>1</sup> No estimation of gaseous hydrogen was made.

*Method of experiment.*

Ten grams of barium hexosediphosphate were ground up with 4.7 g. of sodium sulphate in 250 cc. water. The barium sulphate was filtered off and washed with 50 cc. water. The combined washings and filtrate were now sterilised by filtration through a Chamberland candle and to the solution of sodium hexosediphosphate thus obtained the bacterial suspension was added. To prepare this 2 cc. of a 24-hour bouillon culture of *B. coli* were added to each of ten Roux bottles and incubated for two days. The bacteria were then washed off by using 500 cc. of a sterile solution containing 6 g. potassium sulphate and 0.5 g. magnesium sulphate per litre. Filtered oxygen was bubbled slowly into the liquid contained in a 1500 cc. flask to which was connected an absorption tube containing alkali for the retention of carbon dioxide, and the whole kept at 37° for ten days. At the end of that time washed air was bubbled through vigorously for four hours and the analysis carried through as subsequently described.

In the case of the anaerobic fermentation of the sodium hexosediphosphate, nitrogen was bubbled through the liquid in the digestion flask for two hours before the carbon dioxide absorbing apparatus was attached. The outlet of this was placed below mercury and the whole left in a warm room for one week. At the end of the fermentation period nitrogen was passed through the apparatus for four hours with the delivery tube of the nitrogen well below the surface of the liquid.

*Analytical methods.*

*Carbon dioxide.* In determining the carbon dioxide formed, the amount absorbed by the alkali in the absorbing apparatus was determined by double titration, using methyl orange and phenolphthalein. Some carbon dioxide was at times retained in the combined form in the decomposition mixture as carbonate or bicarbonate; this residual carbon dioxide was determined by taking a portion of the bacterial digest, strongly acidifying with sulphuric acid and aspirating for a couple of hours into alkali.

For the determination of the alcohol and volatile acids, formic and acetic, about 500 cc. of the bacterial digest were acidified with 10 cc. phosphoric acid and distilled with steam. The volatile acids passed over quite slowly so that it was necessary to distil as much as two or three litres, titrating each successive portion of 250 cc. until a small but almost constant titre was obtained, due doubtless to the lactic acid that continued to come over in minute quantities. The separation of the volatile and non-volatile acids was therefore not very accurate.

*Alcohol.* Part of the neutralised distillate was used for the determination of the alcohol; 50 cc. were usually taken, 25 cc. distilled off and the alcohol was estimated in the distillate by the method of Pringsheim [1908]. Care was taken to keep the final volume of the sample oxidised by the dichromate constant and equal to that used in standardising the dichromate, otherwise

the readings were quite irregular. A dilution of 110 cc. was used throughout and at that dilution 1 cc. *N*/20 dichromate was found to oxidise 0.5919 mg. alcohol. As traces of alcohol were sometimes retained by the barium hexosediphosphate and barium monophosphate, the alcohol content of these was estimated by dissolving the barium salts in water or decomposing them by means of sodium sulphate, filtering, distilling and estimating the alcohol in the distillate. A little alcohol was sometimes carried over from the digest into the carbon dioxide absorbing apparatus and this also had to be estimated.

*Volatile acids.* The total neutralised steam distillate was evaporated to dryness on a water-bath and extracted with water and filtered from various wax-like substances invariably present. The formic acid was estimated in this extract by the method of Blank and Finkenbeiner [1898] and the acetic acid by difference from the total titre.

*Succinic and lactic acids.* These were estimated by the method described by Grey [1917].

*Carbohydrate.* Any residual reducing sugar left at the end of the bacterial digest was estimated by precipitating the protein from a sample of the digest with a slight excess of Patein's mercuric nitrate solution, and using Bertrand's method to determine the hexose in the clear filtrate. Usually no residual carbohydrate was found in aerobic fermentation unless the oxygen supply was limited, but under anaerobic conditions as much as 10 % of the hexose was still unfermented, although the hydrolysis of the sodium hexosediphosphate had been nearly complete. The carbohydrate was found not to be a free hexose for the most part but rather a non-reducing polysaccharide, which was estimated by taking 20 cc. of the filtrate, acidifying with 4 cc. dilute sulphuric acid, heating in a boiling water-bath for 1-2 hours, neutralising, and then determining the hexose by Bertrand's method.

*Inorganic and organic phosphorus.* The phosphorus present both as free phosphate and combined in the hexosephosphates was determined by Briggs' modification of the Bell-Doisy method [1922]. This was best done by adding 5 cc. of an 8 % solution of trichloroacetic acid to 10 cc. of the digest and filtering. 10 cc. of the clear filtrate were diluted to 100 cc. and this solution was used for inorganic and organic phosphorus determinations.

During the process of bacterial digestion both aerobically and anaerobically, practically all the hexosephosphates were decomposed so that only a small amount of the phosphorus was found not to be present as inorganic phosphate. From the increase in the inorganic phosphate, the amount of the hexosephosphate that had been decomposed was calculated and from this the carbon for the carbon balance.

*Products of decomposition of sodium hexosediphosphate by B. coli.*

The amounts of the various substances formed by aerobic and anaerobic decomposition were as follows:

Table I.

	Aerobic decomposition		Anaerobic decomposition	
	Weight g.	C atoms per mol. of sugar fermented	Weight g.	C atoms per mol. of sugar fermented
Carbon dioxide ...	3.1930	5.62	0.5632	1.26
Alcohol ...	0.0900	0.30	0.2393	1.03
Acetic acid ...	0.0579	0.15	0.5860	1.92
Formic acid ...	0.0021	0.003	0.1352	0.29
Lactic acid ...	0.0218	0.06	0.0478	0.16
Succinic acid ...	0.0102	0.03	0.3960	1.32
Residual carbohydrate	—	—	0.3200	—
	3.3750	6.163	2.2875	5.98
Original carbohydrate	2.3230	—	2.1460	—

*Preparation of barium hexosemonophosphate.*

The combined filtrates from the extraction of the crude barium hexosediphosphate were united and treated with a solution of basic lead acetate until precipitation was complete. The lead was removed by suspending the precipitated lead salts in water and treating with hydrogen sulphide. To the filtrate from the lead sulphide, hot baryta and an equal volume of alcohol were added to precipitate the crude barium hexosemonophosphate.

To purify the barium hexosemonophosphate it was extracted with 10 % alcohol, filtered, and reprecipitated with an equal volume of alcohol, washed frequently with absolute alcohol and dried *in vacuo*. On analysis of 1 g. of this product, which dissolves quite easily in water, giving a clear solution, its glucose equivalent was found to be 0.2720 g. There was no trace of inorganic phosphate and the combined phosphorus was 0.0709 g. (calculated 0.0785).

*Decomposition of sodium hexosemonophosphate by B. coli.*

This partially purified product was converted into sodium salt and subjected to the action of the *B. coli* under aerobic and anaerobic conditions in the way employed in the experiments with the hexosediphosphate. For the aerobic decomposition 9 g. of barium hexosemonophosphate were employed and for the anaerobic decomposition 6 g.

Table II.

	Aerobic decomposition.		Anaerobic decomposition.	
	Weight (g.) per 1 g. of Ba salt used		Weight (g.) per 1 g. of Ba salt used	
Carbon dioxide ...	...	0.184	...	0.117
Alcohol ...	...	0.028	...	0.052
Acetic acid ...	...	0.152	...	0.085
Formic acid ...	...	0.005	...	0.009
Lactic acid ...	...	0.004	...	0.056
Succinic acid ...	...	0.002	...	0.038
Residual carbohydrate ...	...	0.019	...	0.024

In each case there was some residual carbohydrate unfermented and a small amount of phosphorus still in organic combination.

From the above tables it appears that both with the mono- and the di-phosphates oxygenation results in a marked decrease in the formation of the acids as well as of the alcohol, and a corresponding increase in carbon dioxide.

The high acetic acid content of the products of aerobic fermentation of sodium hexosemonophosphate would probably have been markedly diminished and the carbon dioxide formed increased if the oxygen supply had been more rapid. Acetic acid may possibly be one of the last stages in the decomposition of hexose by *B. coli*. In this regard the results are not similar to those found by Grey for the action of *B. coli* on glucose, who states that in this case the effect of introducing oxygen in the fermentation is to increase the lactic, acetic and succinic acids and to diminish the hydrogen, carbon dioxide and formic acid but to leave the alcohol unchanged. On the other hand the decomposition under anaerobic conditions is of the same character as that of the free sugars.

#### REFERENCES.

- Blank and Finkenbeiner (1898). *Ber. deutsch. chem. Ges.* **31**, 2980.  
Briggs (1922). *J. Biol. Chem.* **53**, 13.  
Grey (1917). *Biochem. J.* **11**, 134.  
—— (1919). *Proc. Roy. Soc. Lond. B*, **90**, 75, 92.  
—— (1920). *Proc. Roy. Soc. Lond. B*, **91**, 294.  
Grey and Young (1921). *Proc. Roy. Soc. Lond. B*, **92**, 135.  
Pringsheim (1908). *Biochem. Z.* **12**, 155.  
Robison (1922). *Biochem. J.* **16**, 809.



# L. A NOTE ON THE PRECIPITATION OF THE ANTISCORBUTIC FACTOR FROM LEMON JUICE.

BY SYLVESTER SOLOMON ZILVA.

*From the Biochemical Department, Lister Institute, London.*

*(Received February 28th, 1927.)*

THE method of fractionation of the antiscorbutic factor introduced by the author [1924, 1925, 1] is based on the facts that the active principle is precipitated by basic lead acetate and that inactive impurities after the removal of the acids from the juice are eliminated by precipitation with neutral lead acetate and alcohol. The main objection to this procedure is that the addition of basic lead acetate in excess brings the reaction of the resulting solution to the alkaline side of neutrality and thus a certain risk of inactivation is incurred. Evidently the stage at which the vitamin is precipitated by the lead acetate is controlled by the hydrogen-ion concentration of the medium [Zilva, 1925, 2], and it is now found that the major part of the activity is in the fraction precipitated at about  $p_H$  7. This is seen from the following experiment. Lemon juice was neutralised with calcium carbonate and allowed to stand for about an hour, after which time it was filtered. An excess of neutral lead acetate was then added to the filtrate, and the reaction adjusted by the addition of dilute ammonia to  $p_H$  7 (phenol red). The precipitate was finally centrifuged and decomposed in the usual way. This preparation was tested out on guinea-pigs (250–300 g.) by the daily administration of freshly prepared doses equivalent to 1.5 cc., 3 cc. and 5 cc. of the original lemon juice, three animals being used for each dose. On the lowest dose only one animal survived the test term of 60 days, showing scorbutic signs at the autopsy after being chloroformed; of the other two animals one died after 56 days and the other after 52 days. The guinea-pigs on the 3 cc. and 5 cc. doses were chloroformed after 60 days, and only in the former group were *very* slight signs of scurvy found at the *post-mortem* examination. It is, therefore, seen that the best part of the active principle was precipitated by the lead acetate at  $p_H$  7. Traces of the antiscorbutic factor could be demonstrated in the filtrate by testing the precipitate obtained on raising the  $p_H$  of the solution to 7.5.

Further investigation has shown that most of the antiscorbutic factor is brought down by lead acetate within the  $p_H$  range of 5.4–7.2. Decitrated lemon juice as prepared above was treated with an excess of neutral lead acetate. After centrifuging the precipitate, the  $p_H$  of the supernatant solution

(5.4) was raised by the cautious addition of dilute ammonia to  $p_H$  7.2. A further precipitate much smaller in bulk was formed, which, on being decomposed, was tested for its activity in the same doses and on the same number of animals as above. The activity was found to be of the same order.

As will be seen from the following table, in spite of the undiminished antiscorbutic activity there is a great reduction in the total solids and in the sugar-content of this preparation. This fraction which is quickly and conveniently prepared can therefore be utilised as a suitable starting-point for further purification.

Table I.

Fraction composed of the total precipitate at $p_H$ 7		
No.	Dry matter (%)	Reducing power as glucose (%)
1	1.610	0.580
2	2.432	0.930
3	2.136	0.800
Corresponding fraction obtained by precipitation between $p_H$ 5.4-7		
No.	Dry matter (%)	Reducing power as glucose (%)
1	0.1278	0.041
2	0.1800	0.070
3	0.199	0.053

The author wishes to express his thanks to the Medical Research Council for a whole-time grant.

## REFERENCES.

- Zilva (1924). *Biochem. J.* **18**, 632.  
 — (1925, 1). *Biochem. J.* **19**, 589.  
 — (1925, 2). *J. Soc. Chem. Ind.* **44**, 445.

# LI. THE PROTEIN ERROR IN ESTIMATING $p_H$ WITH NEUTRAL RED AND PHENOL RED.

BY ELIZABETH HERDMAN LEPPER  
AND CHARLES JAMES MARTIN.

*(From the Department of Experimental Pathology, Lister Institute, London.)*

*(Received March 1st, 1927.)*

SØRENSEN [1910, 1912] first systematically studied the protein error of the indicators then available, including neutral red. The solutions of protein contained sodium chloride and a phosphate buffer, and the  $p_H$  was estimated electrically and colorimetrically. He found that 2 % gelatin had no colorimetric error, 2 % peptone gave readings about 0.13  $p_H$  too low and 2 % crude egg-white 0.6 to 0.7 too low.

Homer [1917], using the undiluted sera of horses immunised against diphtheria containing 4 to 8 % of proteins, found that the colorimetric  $p_H$  with neutral red was in the majority of cases 0.02 to 0.11 too low, but that occasionally it was as much as 0.2 too high. Phenol red under the same conditions gave colorimetric results which were always 0.84 to 0.92 too low, the error being greater with the more alkaline sera. Atkin (private communication), using horse-serum diluted five times with 0.9 % saline, found that the colorimetric error with phenol red was 0.17 at  $p_H$  6.74 and 0.41 at  $p_H$  8.33. Cullen [1922] found that human plasma had to be diluted twenty times before the colorimetric  $p_H$ , as estimated with phenol red, agreed with the electrometric determination. A one-in-three dilution of the plasma determined colorimetrically gave 0.4  $p_H$  lower than the electrical measurement.

In the course of some experiments connected with the concentration of horse-sera containing antitoxin, we had occasion to examine the error of the colorimetric determination of the  $p_H$  of a strong solution of horse-pseudoglobulin when phenol red and neutral red were used as indicators. The results obtained were so different from those which we had previously observed when using these indicators for the colorimetric determination of the  $p_H$  of whole serum that they suggested that pseudoglobulin and albumin behaved differently. We accordingly carried out a parallel series of observations with serum-albumin of the horse which had been six times recrystallised. We found that with both these indicators the error caused in the colorimetric determination of  $p_H$  by pseudoglobulin was in the opposite direction to that caused by serum-albumin. Serum-albumin diminished the colour of the indicators; pseudoglobulin increased it. The divergence between colorimetric

and electrometric is, as might be anticipated, in all cases opposite with neutral red to that with phenol red.

These results led us to examine also the effect of egg-white and recrystallised egg-albumin.

#### OBSERVATIONS ON NEUTRAL RED.

The  $p_H$  of a solution of pseudoglobulin, twice fractionated with  $(NH_4)_2SO_4$ , containing 16.5 % protein, was determined electrically. The solution was not entirely free from euglobulin. It was titrated electrometrically to the isoelectric point. From the  $N/10$  acid required the amount of base in the globulin solution was calculated. Some  $M/45$  phosphate having the same  $p_H$  electrically

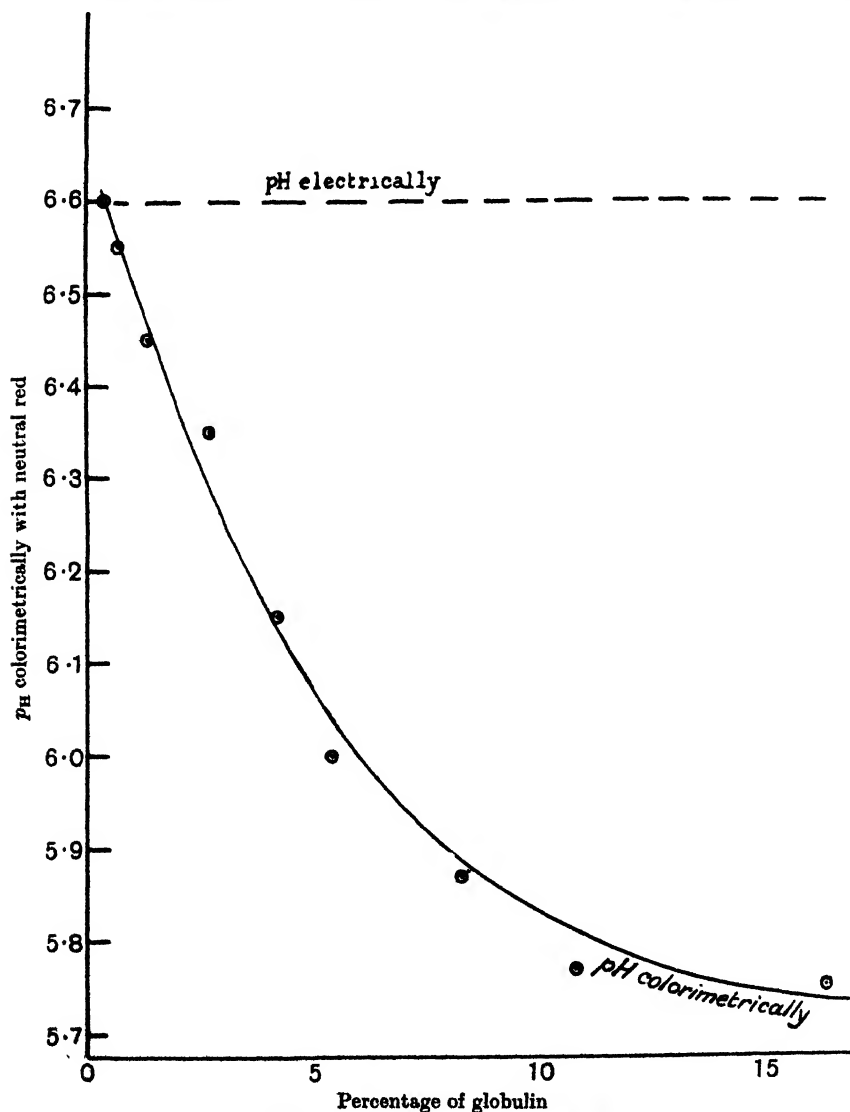


Fig. 1. Globulin error of neutral red.

as the globulin solution was prepared. This contained the same concentration of Na as the globulin solution. Standard phosphate tubes at intervals of 0.1  $p_H$  containing neutral red as indicator were used for the colorimetric determinations.

Various dilutions of the globulin solution were made by adding the phosphate solution. The results are shown in Table I and graphically in Fig. 1.

Table I. *The pseudoglobulin error of neutral red.*

Globulin %	$p_H$ colorimetrically	Globulin %	$p_H$ colorimetrically
16.35	5.75	2.7	6.35
10.8	5.77	1.35	6.45
8.17	5.87	0.67	6.55
5.4	6.0	0.33	6.60
4.09	6.15	0.17	6.60
		0.0	6.60

A similar experiment was carried out with recrystallised serum-albumin. In this case the strongest solution contained 3 % albumin,  $M/60$  phosphate and 0.85 % sodium chloride. It was diluted with  $M/60$  phosphate which contained the same concentration of sodium and had the same  $p_H$  as that found electrically for the albumin solution. The results are shown in Table II and Fig. 2. The effect is in the opposite direction to that with globulin. The serum error of neutral red will therefore depend on the proportion of albumin to globulin in any particular sample.

Table II. *The serum-albumin error of neutral red.*

Albumin %	$p_H$ colorimetrically	Albumin %	$p_H$ colorimetrically
3.0	6.93	0.095	6.45
1.5	6.86	0.047	6.35
0.75	6.75	0.23	6.35
0.38	6.65	0.0	6.35
0.19	6.55		

Ledingham [1907] found that in horses immunised against diphtheria the globulin content of the serum increased. The serum of one horse at the beginning of treatment contained 5 % globulin and 2.7 % albumin. This serum would, from our curves, have a colorimetric error of 0.58 less 0.60 so that the  $p_H$  would be 0.02 too low. Six weeks later this horse's serum contained 8 % globulin and 2 % albumin and the colorimetric error would now have been 0.51 less 0.74 or 0.23 too low.

We next tested the effect of purified egg-albumin and found to our surprise that a 7 % solution of six times recrystallised egg-albumin showed no colorimetric error. Crude egg-white, however, 20 % by volume, gave a  $p_H$  0.52 too low. Sørensen [1910] with 2 % egg-white found the  $p_H$  colorimetrically 0.6 to 0.7 too low.

#### OBSERVATIONS WITH PHENOL RED.

In these experiments the protein was always made up with  $M/60$  phosphate and enough sodium chloride added to bring the molecular concentration of sodium to approximately  $M/7$ , for we have shown [Lepper and Martin, 1926] that the dissociation of phenol red is sensitive to cations and in order to get identical results electrically and colorimetrically when using  $M/15$  phosphate

solutions as standards it is necessary to have the molecular concentration of the sodium in the solution to be tested approximately the same as in the standards. The dilutions were made with  $M/60$  phosphate containing sodium chloride adjusted colorimetrically to the  $p_H$  required.

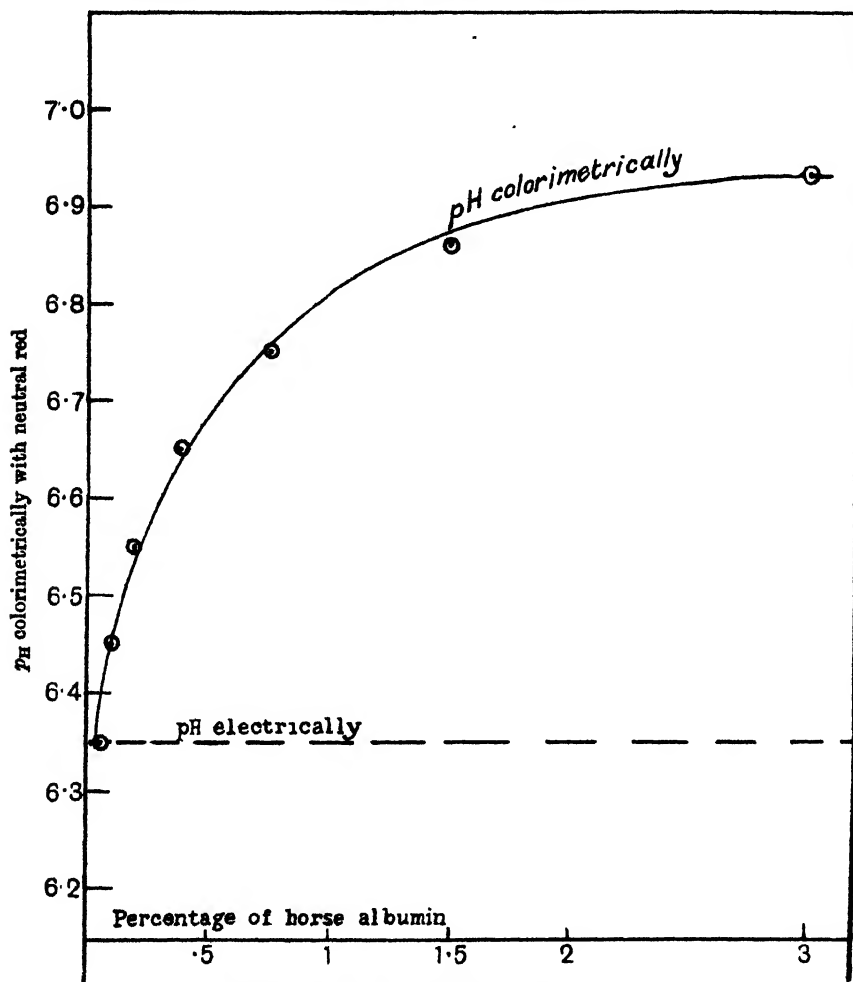


Fig. 2. The albumin error of neutral red.

The results with globulin are shown in Table III. Pseudoglobulin causes a slight rise in the apparent  $p_H$  which is more easily detected at the alkaline end of the indicator range. 2 % has no effect.

Table III. The pseudoglobulin error of phenol red.

Globulin %	$p_H$ colorimetrically	Globulin %	$p_H$ colorimetrically
11.0	8.05	12	7.42
7.0	8.02	8	7.41
3.5	7.95	4	7.40
1.75	7.93	2	7.38
0.0	7.93	0	7.38

Table IV. *The serum-albumin error of phenol red.*

Albumin %	$p_H$ colorimetrically	Albumin %	$p_H$ colorimetrically
3	7.69	0.125	7.99
2	7.73	0.0625	8.0
1	7.80	0.031	8.02
0.5	7.86	0.0	8.02
0.25	7.94		

Albumin, however, produces a marked decrease in the apparent  $p_H$  as shown in Table IV and Fig. 3; this was slightly greater the higher the  $p_H$ . With serum the albumin error will, therefore, always predominate. Cullen [1922] with human serum and a bicarbonate buffer found a higher colorimetric error than we have done with serum-albumin of the horse.

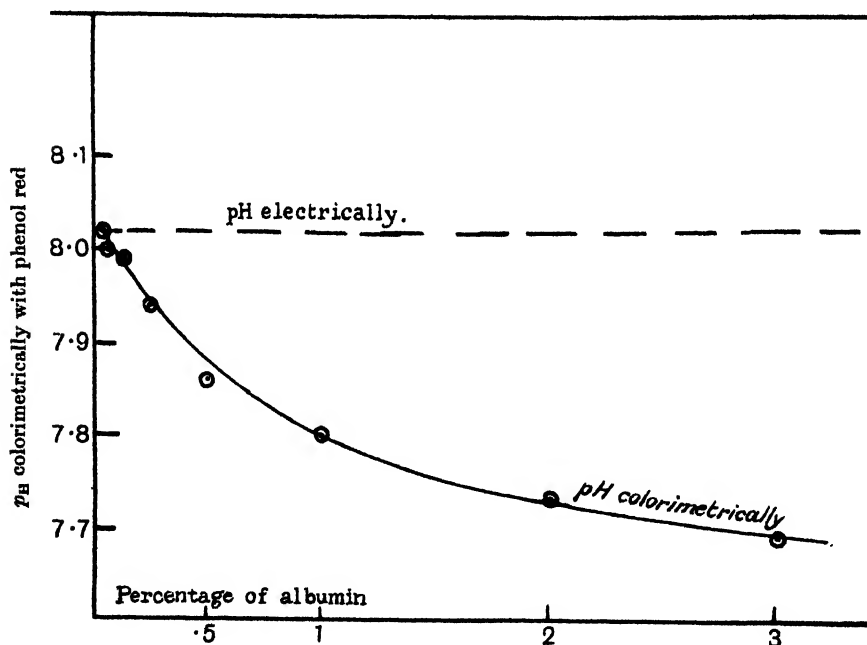


Fig. 3. The albumin error of phenol red.

The presence of pure egg-albumin again caused no error but crude egg-white, 20 %, decreased the  $p_H$  by 0.1.

#### DISCUSSION.

The results obtained show that the protein error of an indicator must be determined for each protein. The effect of serum, for instance, is a summation of the opposed effects of the albumin and globulins. These may almost balance each other, as happens in the case of neutral red, or one error may predominate as in the case of phenol red. Proteins belonging to the group of albumins but derived from different sources do not react in the same way with a particular indicator; with neutral red the error is considerable with

horse-albumin but negligible with egg-albumin. The experiments do not throw any light on the way in which proteins interfere with the colorimetric determination of  $p_H$ . With the indicators used, there was no precipitation of the dye or alteration in colour such as occurs, for instance, with bromo-o-cresolsulphonaphthalein which gives a blue colour in the presence of proteins impossible to match with the standards. The diminution of colour of both types of indicator in the presence of albumins might be due to diminished dissociation or adsorption on the colloid particles, but an increase in the presence of globulins requires the assumption of complexes between the dye and protein possessing a higher dissociation coefficient.

#### SUMMARY.

The protein errors with neutral red and phenol red for pseudoglobulin and serum-albumin of the horse and for egg-albumin and egg-white in varying concentrations, have been determined.

With both these indicators the colorimetric error due to the presence of this pseudoglobulin is in the opposite direction to that caused by serum-albumin. In either case the errors with neutral red are in the opposite sense to those with phenol red.

Pure egg-albumin up to 7 % does not cause any colorimetric error with either neutral red or phenol red.

#### REFERENCES.

- Cullen (1922). *J. Biol. Chem.* **52**, 501.
- Homer (1917). *Biochem. J.* **11**, 283.
- Ledingham (1907). *J. Hygiene*, **7**, 5.
- Lepper and Martin (1926). *Biochem. J.* **20**, 45.
- Sørensen (1910). *Compt. Rend. Carlsberg*, **8**, 1.
- (1912). *Ergebn. Physiol.* **12**, 393.



## LII. ON THE ABSORPTION OF VITAMIN D FROM THE SKIN.

BY ELEANOR MARGARET HUME, NATHANIEL SAMPSON LUCAS,  
AND HANNAH HENDERSON SMITH.

*From the Department of Experimental Pathology, Lister Institute, London.*

*(Received March 1st, 1927.)*

AN animal can either obtain the necessary supply of vitamin D for skeletal growth from its food or it can make it for itself from some precursor, if its surface is sufficiently exposed to sunlight or ultra-violet rays.

The principle which is activated to form vitamin D has hitherto been found associated with cholesterol, but from observations of Rosenheim and Webster [1926; 1927, 1, 2] and Heilbron, Kamm and Morton [1927] the precursor of vitamin D is not ordinary cholesterol, although it clings to it during successive recrystallisations. Rosenheim and Webster [1927, 2] adduce good reasons for supposing that it is ergosterol. They find that ergosterol is 2000 times as rich in the provitamin as brain cholesterol. Ergosterol unlike ordinary cholesterol has three double bonds and has three absorption bands in the ultra-violet, the strongest being at a wave length of  $280\ \mu\mu$ , accompanied by two others of less intensity, at wave lengths of about  $270\ \mu\mu$  and  $295\ \mu\mu$ . Hess and Weinstock [1925] have shown that "cholesterol" irradiated by ultra-violet rays exerts its anti-rachitic action when injected subcutaneously as well as when taken by the mouth. They also found [Hess, 1925; Hess and Weinstock, 1925] that pieces of irradiated skin from infants and calves exercised an anti-rachitic action when eaten by rats. Hess, Weinstock and Helman [1925] showed that lanolin contains the precursor of vitamin D and suggested that it is by the activation of the cholesterol in the layers of the epidermis and its subsequent absorption that animals are protected from rickets. Yet another possibility is that the precursor is activated whilst coursing through the superficial capillaries of the skin, but for this to occur, sufficient radiant energy of the required wave length must penetrate the 0.1 to 0.2 mm. of the epidermis covering the capillaries. The possession by ergosterol of a strong absorption band about the wave length of  $280\ \mu\mu$  [Rosenheim and Webster, 1926] indicates that light of this wave length would be particularly effective in activating it. The epidermis, however, is very opaque to this wave length. One of us (N. S. L.) has not succeeded in obtaining any indication of the  $280\ \mu\mu$  line in a spectrogram of a quartz mercury vapour lamp, distant 2 feet, after 3 hours' exposure, through 0.1 mm. of epidermis when using an Ilford Process plate (H. and D. = 50).

Radiations of wave length  $280\text{ }\mu\mu$  are not present to a significant extent in sunlight which has passed through the upper atmosphere [Cornu, 1878, 1879, 1880, 1890; Miethe and Lehman, 1909; Dember, 1912; Wiegand, 1913] so that the precursor of vitamin D must be capable of being activated, if more slowly, by ultra-violet light of greater wave length impinging on the body. For ultra-violet light of slightly greater wave length, the epidermis is much less opaque. Calculating from Hasselbalch's [1911] observations,  $1/56$  of the incident light of a wave length of  $297\text{ }\mu\mu$  penetrates  $0.1\text{ mm.}$  and  $1/3000$  penetrates  $0.2\text{ mm.}$  of epidermis. At present we are ignorant of the amount of radiant energy required to activate sufficient of the precursor to provide an adequate supply of vitamin D for an animal, but it would appear to be very small, when of the appropriate wave length; for  $1\frac{1}{2}$  minutes' daily exposure to a mercury vapour quartz lamp is sufficient to prevent rickets in an albino rat, for the most part covered with fur, and fed on a rickets-producing diet [Hess, 1922]. It was observed by Chick and co-workers [1923] that rickets in a child was healed when the hand and fore-arm only were exposed to ultra-violet irradiation. Hess and Unger [1921] made a similar observation, but in this case the exposure was to the rays of the sun.

The accompanying experiments were designed to test whether irradiated cholesterol, applied to the external surface, could exercise its anti-rachitic action through the skin.

#### TECHNIQUE.

Two methods were used for testing the potency of the various anti-rachitic measures tried. For the first method, young rats were used, which at about  $50\text{ g.}$  weight had been placed upon a diet deficient in both fat-soluble vitamins; the method, which has been described before [Hume and Smith, 1926], takes advantage of the fact that the animal's reserve of vitamin D is exhausted before that of vitamin A. After the rats had been on this diet for 4 weeks the treatment to be tested was applied, and the degree to which growth was restored was taken as a measure of the anti-rachitic potency of the treatment.

For the second method, young rabbits, between  $300$  and  $500\text{ g.}$  weight, were used and they were fed on the diet No. 3143 of McCollum [1921], which is low in phosphorus. They also received on week days  $5\text{ cc.}$  of orange juice, given with a pipette; it could not be mixed with the diet, as this was given dry. On this diet rabbits develop gross lesions of rickets, unless anti-rachitic treatment of some sort is applied. In the first experiment the rabbits were kept on the diet for 8 weeks and in the second for 5 weeks. At the end of the experiment a femur from each rabbit was ashed, after extraction with ether and alcohol. The histology of the rib junctions was also studied.

The part of the body chosen, as most inaccessible to the animal itself, for the application of irradiated cholesterol was the middle of the chest in rats and the back of the neck in rabbits. The skin was kept as clear of hair as

possible by depilation and cutting of the hair. Depilation was at first carried out with barium sulphide, but in the experiment with rats and in the first experiment with rabbits, it caused slight soreness, so the experiment with rabbits was repeated using toilet "Veet," in which the barium sulphide is diluted with a cream; no soreness then resulted.

About 0.2 g. of cholesterol was dissolved in ether and evaporated on a glass slide in order to provide a thin layer. This was irradiated for 10 minutes, at a distance of 16 inches from a mercury vapour quartz lamp (Ulviarc), freshly for every application, and the application was made three times a week. The cholesterol was gently applied to the skin with the point of a finger, over an area of about one square inch, and a little hardened cotton-seed oil was afterwards applied to make the cholesterol adhere. The animals were dressed in chamois-leather jackets, fastening down the back, which prevented them from gaining access in any way to the treated patch of skin and each animal was kept in a separate cage. It was found extremely difficult to be perfectly certain that no particle of irradiated cholesterol should accidentally reach the animals' mouths, and the experiment was performed three times, each time with added precautions, but each time with the same result.

Table I. *Growth of rats on a diet deficient in fat-soluble vitamins; irradiated cholesterol applied to the skin from 27th-47th day.*

Treatment				No. of rat	Sex	No. of litter	Weight on 27th day g.	Weight on 47th day g.	Total increase g.
Control	...	...	...	L 1	♀	310	52	60	8
				L 3	♂	"	68	78	10
				L 4	♂	"	71	84	13
				L 2	♀	310	51	73	22
Irradiated cholesterol applied to skin				L 5	♂	"	67	86	19
				L 6	♂	"	66	81	15

*Exp. 1.* In the first experiment, six white rats of the Wistar Institute breed, all belonging to the same litter, were used. They all received the diet, deficient in fat-soluble vitamins, already referred to. After 27 days on the diet, irradiated cholesterol was applied to a depilated patch on the chests of three of the rats, three times a week for 20 days. The remaining three were left untreated as controls. Table I shows the growth of the two sets of animals, over the twenty days of treatment. The three treated animals show growth which is about double that of the untreated animals and it would therefore appear that by inunction the irradiated cholesterol has been able to exercise a definite effect. It was however felt that by using a larger animal it would be considerably easier to take the necessary precautions against the entry of the anti-rachitic agent, by any other channel than through the intact skin. It was therefore decided to use rabbits.

*Exp. 2.* Five rabbits, all stated to belong to one litter, were used. They all received the diet, McCollum 3143. Two received no further treatment; irradiated cholesterol was rubbed into the shoulders of two 3 times a week,

and one was irradiated directly, with a mercury vapour quartz lamp for 10 minutes daily, at a distance of 46 cm. Treatment lasted for 8 weeks. Table II A, which gives the results, shows that both histologically and in their percentage ash the bones of the animals which had had cholesterol applied to their skins approached those of the animal irradiated directly, while the controls showed rickets histologically and a considerably lower percentage of ash.

The two animals which received cholesterol, were, however, at the end of the experiment, slightly sore and it was also felt that in order to imitate any natural absorption which might take place, the irradiated cholesterol should have been gently stroked on the skin, and not rubbed in as had so far been done. The next experiment was also planned to include rabbits irradiated directly, over a small area of the body only.

*Exp. 3.* In Exp. 3, seven rabbits, stated to belong to two litters, were used. Depilation was carried out by means of "Veet." The diet was the same as in the last experiment, but treatment was only continued for 5 weeks. Two of the rabbits were used as untreated controls. On two, a patch was depilated at the back of the neck, and three times a week irradiated cholesterol was very gently stroked on to the skin and a little hardened cotton-seed oil was lightly smeared over it. The application was only made to a small area of about one square inch and it was felt during the experiment that, if the treatment in so slight a form could produce an effect, it must be very potent. From Table II B it will be seen that a very definite effect was produced and though the results obtained are not quite as good as in the case of animals irradiated directly, yet they are very much better than the results yielded by the controls, one of which showed an extraordinarily low ash in the bone.

Table II. *Histological report on the rib junctions and percentage ash in the extracted bone of rabbits, fed on diet 3143 of McCollum, and treated with irradiated cholesterol applied to the skin, or with direct irradiation of whole or part of the body.*

Treatment	No. of rabbit	Sex	No. of litter	% ash in extracted bone	Histological report
A. Control ...	2	♀	1	46.8	Severe rickets
	5	♀	"	48.1	"
Irradiated cholesterol applied to skin	3	♂	"	56.3	No rickets
	6	♂	"	55.7	"
Irradiated directly ...	4	♂	"	58.0	"
B. Control ...	8	♂	2	46.2	Moderate rickets
	13	♀	3	37.1	Very severe rickets
Irradiated cholesterol applied to skin	7	♀	2	52.9	No rickets
	10	♂	3	52.5	Slight rickets
Irradiated through hole 2.5 × 3.5 cm.	9	♂	2	56.4	—
	11	♂	3	55.6	—
	12	♂	3	57.0	—

The three remaining rabbits were irradiated directly through a small hole in a black cloth. The cloth was of black sateen doubled. Penetration of light

through the cloth was tested by means of a spectrograph; the spectrogram showed no penetration by ultra-violet rays, after half an hour's exposure to a mercury vapour arc. The hole in the cloth measured 2.5 by 3.5 cm. The rabbit was rolled in the black cloth and a depilated area on its back was exposed directly through the hole, at a distance of 50 cm. to a mercury vapour quartz lamp. The exposure lasted 10 minutes and took place three times a week. Approximately, but not quite, the same area of skin was irradiated at each exposure. With one rabbit, it was attempted to cleanse the depilated area with ether and free it from cholesterol, but this was found to be quite impossible and the rabbit, No. 12, is therefore included with Nos. 9 and 11, in the group of those irradiated directly through a hole.

All the rabbits in Exp. 3 were killed when they were slightly younger than the rabbits in Exp. 2, so that a slightly lower range of values for the percentage ash might be expected in Exp. 3. Allowing for this it is apparent that calcification in an animal, of which only a small area has been irradiated periodically for 10 minutes, is only slightly inferior to that of an animal whose whole body has been irradiated for the same time.

#### CONCLUSIONS.

1. Vitamin D in irradiated cholesterol can be absorbed from a small area of undamaged skin in sufficient amount to supply the needs of the animal.

2. The suggestion of Hess that activation of lanolin by sunlight and its subsequent absorption through the skin is a possible source of this vitamin is supported by experiment.

3. Rickets was prevented in rabbits fed upon a rickets-producing diet, and almost normal calcification of the bones produced, when an area of skin  $2.5 \times 3.5$  cm. was irradiated for ten minutes three times a week.

The writers' thanks are due to Prof. C. J. Martin for kind help and advice, to the Lister Institute for its hospitality, and to the Medical Research Council for a whole time and a half time grant for salaries of two of the workers.

#### REFERENCES.

- Chick and co-workers (1923). *Med. Res. Council Special Report Series*, No. 77.  
Cornu (1878). *Compt. Rend. Acad. Sci.* **88**, 1101, 1285.  
— (1879). *Compt. Rend. Acad. Sci.* **89**, 808.  
— (1880). *Compt. Rend. Acad. Sci.* **90**, 940.  
— (1890). *Compt. Rend. Acad. Sci.* **111**, 941.  
Dember (1912). *Abhand. Nat. Wiss. Ges. Isis, Dresden*, **2**, 196.  
Hasselbalch (1911). *Skand. Arch. Physiol.* **25**, 55.  
Heilbron, Kamm and Morton (1927). *Biochem. J.* **21**, 78.  
Hess (1922). *J. Amer. Med. Assoc.* **78**, 1177.  
— (1925). *J. Amer. Med. Assoc.* **84**, 1910.  
Hess and Unger (1921). *J. Amer. Med. Assoc.* **77**, 39.

- Hess and Weinstock (1925). *J. Biol. Chem.* **64**, 181.  
Hess, Weinstock and Helman (1925). *J. Biol. Chem.* **63**, 305.  
Hume and Smith (1926). *Biochem. J.* **20**, 340.  
McCollum, Simmonds, Shipley and Park (1921). *J. Biol. Chem.* **47**, 507.  
Miethe and Lehman (1909). *Ber. Berlin. Akad.* 208.  
Rosenheim and Webster (1926). *Biochem. J.* **20**, 537.  
—— — (1927, 1). *Biochem. J.* **21**, 27.  
—— — (1927, 2). *Lancet*, i, 306.  
Wiegand (1913). *Physiol. Zentr.* **14**, 1144.

# LIII. A SEARCH FOR VITAMIN D IN THE DIATOM *NITZSCHIA CLOSTERIUM* (W. SM.).

By JOAN LEIGH LEIGH-CLARE.

*From the Department of Experimental Pathology, Lister Institute, London.*

*(Received March 1st, 1927.)*

JAMESON, DRUMMOND and COWARD [1922] showed that the marine diatom *Nitzschia closterium* (W. Sm.) *forma minutissima*, grown in sterilised sea-water with the addition of Miquel's nutrient solutions, can synthesise large amounts of fat-soluble vitamin, and they came to the conclusion that the ultimate though indirect source of the fat-soluble vitamin was this and other marine unicellular organisms.

In view of the recent differentiation of the fat-soluble vitamins, it was suggested to me by Dr H. Chick that it would be interesting to see whether *Nitzschia closterium* also contained vitamin D.

It has been found that the green land plants examined up to the present contain very little, if any, vitamin D [Goldblatt and Zilva, 1923; McClendon and Shuck, 1923; Zucker and Barnett, 1923; Hess and Weinstock, 1924; Bethke, Kennard and Kik, 1925; Chick and Roscoe, 1926; Boas, 1926; Roscoe, 1927] and it was thought that it would be of interest to ascertain whether the same held for marine unicellular organisms containing chlorophyll. There is good reason to believe that these are the ultimate source, via plankton, copepods and small fish, whence deep-sea fish, such as the cod, indirectly obtain their supplies of vitamin A; but it is not known whether the store of vitamin D, found in the liver oils of the codfish, is obtained in this way.

## TECHNIQUE.

The experiments described below were carried out on four different litters of rats. The basal diet given was McCollum's No. 3143 low phosphorus rickets-producing diet [1921].

A culture of *Nitzschia closterium* was kindly supplied to me by Professor Drummond, of University College, London, and from this further cultures were grown as described by Jameson, Drummond and Coward [1922]. At first the cultures were kept in Erlenmeyer flasks which were placed in a window facing north. They were never exposed to direct sunlight and all the light which reached them had to pass through a pane of plate-glass and the glass

wall of the flask. Very little ultra-violet light of wave-length less than  $340\text{ }\mu\mu$  succeeded in reaching the *Nitzschia* in the flasks. It was therefore decided to try to grow the diatom under conditions more comparable to those to which it is exposed when floating in the superficial layers of the sea, and to grow it in shallow glass dishes on a flat roof to catch all the sunlight available, shielding it from bacterial contamination by a sheet of "windowlite."

A spectrogram of the windowlite used was kindly made for me by Dr Lucas. This showed that windowlite has a fair transparency for ultra-violet light of wave-length longer than  $290\text{ }\mu\mu$  or nearly to the extreme range of the sun's spectrum. A padding of sterilised cotton-wool was introduced between the edge of the loose-fitting lid and the rim of the glass vessel in order to preserve the culture from contamination. The *Nitzschia* grew well under these conditions throughout the months of September and October.

Each litter of young rats received the dose of *Nitzschia* after somewhat different treatment. At first, the culture was filtered to dryness on a layer of starch in Gooch crucibles or Büchner funnels, and then further dried for two hours before administration, being mixed with starch in the proportions of 1 to 3. Later the drying at  $37^{\circ}$  was omitted, and in the final experiment the *Nitzschia* was given without any starch. In this last case, the diatom was filtered freshly every day from the glass dishes on the roof.

The amount of water lost, when *Nitzschia* was collected on a Büchner funnel, and dried at  $100^{\circ}$  to constant weight, was from 70-75%. The actual weight of dried material administered would therefore be one-quarter of that of the fresh diatom. The doses specified below refer to the fresh weight of the diatom, after suction on the Büchner funnel.

The *Nitzschia* was always given before the basal diet. It was eaten readily after the first few days. Each batch of the diatom was examined microscopically before filtering to make sure both that the culture was free from other diatoms and also that the diatom was alive and in a healthy condition.

To test the condition of the skeletons of the rats the junctions of the 6th and 7th ribs on the right side were examined histologically, and chemical analyses were made on the leg bones (femora, tibiae and fibulae). The methods used are described in full in the paper by Chick, Korenchevsky and Roscoe [1926].

## RESULTS.

*Exp. 1.* The *Nitzschia* was grown in Erlenmeyer flasks in diffused light passing through plate-glass windows. The culture was filtered on starch, dried at  $37^{\circ}$  and administered with starch as described above.

A litter of five black-and-white rats was put on experiment on June 26th, 1926. The experiment lasted four weeks. Two of the rats, one male and one female, were used as controls; two others, one male and one female, received 0.1 g. (fresh weight) of *Nitzschia* in addition to the basal diet. One rat received 60 mg. of cod-liver oil.



The histological and chemical analyses of their bones, together with the initial weight and growth of the rats, will be found in the table.

Experi- ment	No. of rat	Sex	Dose of <i>Nitzschia</i> g. fresh weight	Initial weight g.	In- crease g.	Duration of exp. (days)	Degree of rickets from histology of rib junctions	Ca % in leg- bones on fat- extracted dry weight
I	7	♂	Control	45	16	26	+	9.7
	5	♂	Control	38	27	26	+	9.4
	10	♂	0.1	41	30	26	+	7.9
	4	♂	0.1	37	23	26	+	7.3
	3	♂	60 mg. C.L.O.	40	25	26	+	13.1
II	16	♂	Control	46	17	19	+	8.0
	17	♂	Control	42	11	20	+	8.4
	12	♂	0.2	42	7	19	+	7.6
	13	♂	0.2	41	14	20	+	8.3
	14	♂	0.1	41	12	19	+	7.1
	15	♂	0.1	39	20	20	+	8.0
III	18	♂	Control 16 days	41	12	16	+	9.8
	21	♂	0.21 after 16 days	41	18	23	+	8.3
	22	♂	Control 18 days	42	10	18	+	10.0
	25	♂	0.21 after 18 days	41	18	25	+	9.3
	20	♂	Control 21 days	44	12	21	+	9.4
	23	♂	0.21 after 21 days	41	25	28	+	9.5
	24	♂	Control 23 days	39	19	23	+	8.9
	19	♀	0.21 after 23 days	39	12	30	+	10.9
IV	26	♀	Control	41	35	21	+	13.0
	27	♀	Control	50	24	21	+	10.6
	29	♀	An average of .378 }	39	34	21	+	10.3
	30	♀	filtered fresh daily }	39	28	21	+	10.1

It will be seen that all the rats except No. 3, which was receiving cod-liver oil, were intensely rachitic; it was impossible to find any significant difference between them histologically. In the chemical analysis it will be seen that, disregarding the animal receiving cod-liver oil (No. 3), the bones of the control animals (Nos. 7 and 5) had a higher percentage of calcium than those of the animals receiving the supplementary rations of *Nitzschia*.

*Exp. 2.* The *Nitzschia* given had been grown in Erlenmeyer flasks for six weeks in the same manner as in *Exp. 1*. At the end of this period it was put into the shallow glass dishes and exposed on the roof, as described at the beginning of this paper. The diatom was filtered off on to starch, dried and administered as in *Exp. 1*.

A litter of six white rats was used. The experiment was started on Sept. 17th, 1926. Two of the rats, a male and a female, were used as controls. These received only the basal diet. Two were given 0.2 g. of *Nitzschia* and two 0.1 g. in addition to their basal ration.

The experiment lasted 19-20 days. There was no striking difference in the calcium content of the bones of the animals, though in each case the bones of the control rats had higher calcium-content than those of the rats of the same sex receiving *Nitzschia*. The histological examination showed little difference between the various animals. They were all rachitic.

*Exp. 3.* The *Nitzschia* was propagated at first indoors and transferred to the roof after three weeks. It remained on the roof for a week or more

before being filtered off. It was given as before, mixed with starch in the proportion of 1 in 4.

A litter of eight black-and-white rats was used. The experiment was commenced on Sept. 27th, 1926. Four rats were taken as controls and were killed after 16, 18, 21 and 23 days respectively. Four other rats of corresponding sex received doses of *Nitzschia* for one week after being on basal diet alone for 16, 18, 21 and 23 days respectively. These rats were then killed. There was very little difference in the calcium content of the bones of two groups of animals. Two controls showed a higher calcium content in their bones than the corresponding animals fed with *Nitzschia* for a further week, and two other controls showed a lower content. Histologically, also, there was little to choose. They all had rickets in a marked degree.

*Exp. 4.* The culture of *Nitzschia* was started indoors for a short time and subsequently grown on the roof and filtered fresh daily for administration to the rats.

A litter of 4 black-and-white female rats was put on experiment on October 7th, 1926. Two of these rats were used as controls and two received *Nitzschia* filtered fresh daily. Their daily dose averaged 0.40 g. (fresh weight) during the first, 0.46 g. during the second and 0.27 g. during the third week.

Chemical analysis of the bones showed that the calcium content of the bones of the control animals was not lower than that of the rats receiving freshly filtered *Nitzschia*.

#### DISCUSSION.

From a paper by Chick and Roscoe [1926], it appears that the anti-rachitic potency of cow's milk is very little increased by feeding the animal on green pasture food while the cow itself is kept in a dark stall. It is only when the cow is itself exposed to the rays of the sun that the anti-rachitic value of its milk increases appreciably. Cows fed on cod-liver oil in a dark stall, however, yield a milk of increased anti-rachitic potency [Golding, Soames and Zilva, 1926]. It seems as if it may be possible to trace a parallel in this respect between land and marine animals.

There are at present only two known sources from which the cod could obtain its supplies of vitamin D:

- (1) The activation of the precursor of vitamin D by direct action of the sun's rays on the cod.

- (2) The food it consumes.

It is difficult to suppose that the cod, a deep-sea fish, is ever exposed to enough sunshine to activate a precursor of vitamin D, so presumably it must obtain its supplies from the food which it consumes. The ultimate source of vitamin A in cod-liver oil has been traced by Zilva and Drummond [1922] to unicellular marine organisms. They say: "It is not directly from these organisms that the cod receives its vitamin but through several intermediaries such as the copepods and larval decapods and mollusca which are present in

plankton and which feed on these unicellular plants. These in turn are consumed by the capelan and other forms of food of the cod."

From the experiments on *Nitzschia closterium* described in this paper it does not appear probable that unicellular marine organisms are the ultimate source of vitamin D in cod-liver oil, and if this is correct there remain to be examined the plankton and the smaller fish, both of which might conceivably be exposed to sufficient radiant energy of the necessary wave-length for synthesis of vitamin D.

My thanks are due to Professor C. J. Martin, Dr H. Chick and Dr Zilva for kind criticism and help, to Professor Drummond for the culture of *Nitzschia*, to the Medical Research Council for a whole time grant, and to the Lister Institute for the hospitality of its laboratories.

#### REFERENCES.

- Bethke, Kennard and Kik (1925). *J. Biol. Chem.* **63**, 377.  
Boas (1926). *Biochem. J.* **20**, 153.  
Chick, Korenchevsky and Roscoe (1926). *Biochem. J.* **20**, 622.  
Chick and Roscoe (1926). *Biochem. J.* **20**, 362.  
Goldblatt and Zilva (1923). *Lancet*, ii, 647.  
Golding, Soames and Zilva (1926). *Biochem. J.* **20**, 1306.  
Hess and Weinstock (1924). *J. Biol. Chem.* **62**, 301.  
Jameson, Drummond and Coward (1922). *Biochem. J.* **16**, 482.  
McClendon and Shuck (1923). *Proc. Soc. Exp. Biol. Med.* **20**, 288.  
McCollum, Simmonds, Shipley and Park (1921). *J. Biol. Chem.* **47**, 507.  
Roscoe (1927). *Biochem. J.* **21**, 211.  
Zilva and Drummond (1922). *Biochem. J.* **16**, 518.  
Zucker and Barnett (1923). *Proc. Soc. Exp. Biol. Med.* **20**, 375.

## LIV. THE CARBOHYDRATE AND FAT METABOLISM OF YEAST.

### IV. THE NATURE OF THE PHOSPHOLIPINS.

BY CHARLES GASPARD DAUBNEY AND  
IDA SMEDLEY MACLEAN.

*From the Biochemical Department, Lister Institute, London.*

*(Received March 1st, 1927.)*

AFTER yeast has been incubated in a well-oxygenated solution of glucose, fructose or sucrose, its fat-content becomes very considerably increased. If the solution in which the yeast is incubated contains not only sugar but also a mixture of alkali phosphates, the total amount of lipoid (ether-soluble matter) which can be extracted from the yeast after its incubation is double that which can be extracted from the same amount of yeast incubated in a similar sugar solution from which phosphates are absent [Smedley-MacLean and Hoffert, 1923]. After incubation in the sugar-phosphate medium the phosphate-content of the yeast is also greatly increased, the increase depending on the concentration of the *sugar* in the medium. After yeast has been incubated in a solution containing only alkali phosphates, no increase in the phosphate-content of the yeast is observed [Smedley-MacLean and Hoffert, 1924].

This effect of the addition of phosphates is very striking and the inference seems permissible that the phosphates themselves play some part in the transformation of carbohydrate to fat. Evidence was adduced to show that the first stage of this change probably consists in the formation of a hexose-phosphate.

Again, in the complex lipin molecule phosphoric acid is known to occur in combination with fatty acids. The relation of the fatty acid to the lipin molecule is still unknown: the fatty acids may first be formed and then built up into the lipin molecule, or perhaps the whole change from carbohydrate to fatty acid may take place while the various groups are associated together in some complex molecule of which phosphoric acid forms an integral part.

Since the formation of fat from carbohydrate in yeast can be very largely influenced by altering the constituents of the medium in which the yeast is incubated, it seemed important to examine the nature of the yeast phospholipins, the proportion which these bear to the other constituents of the lipoid matter and the changes which take place when the composition of the medium is altered, especially by the addition of phosphates.

*Previous work on yeast lipins.*

As early as 1866, Hoppe-Seyler [1866] showed that an ether-soluble substance was present in yeast containing both nitrogen and phosphorus. Nägeli denied this but Hoppe-Seyler [1879] published a further paper in which he showed that of the substance extracted by ether from yeast the unsaponifiable matter constituted 24.14 % and at least another 14 % consisted of lipin. From this lipin he isolated both choline and glycerophosphoric acid.

Koch [1902] showed that the ratio of methyl groups to nitrogen atoms, instead of being 3 : 1 as it is in lecithin, in the yeast lipin was only 1.3 : 1, a ratio very much more nearly akin to that found in kephalin. Koch argued therefore that choline could not be the chief base of yeast lipin.

Sedlmayer [1903] found choline and isolated palmitic acid but failed to find oleic acid. A preliminary note published by Austin [1924] indicated the presence of both lecithin and kephalin in yeast, since both choline and hydroxyethylamine were detected, thus confirming the previous finding of Koch.

The fatty acids of yeast fat have been examined by a number of observers and oleic, linolic and palmitic acids have been identified with certainty and evidence has been adduced that lauric acid is probably present; small amounts of a higher melting acid, possibly arachidic, have also been found [Hinsberg and Roos, 1904; Neville, 1913; Smedley-MacLean and Thomas, 1920].

*Method of experiment.*

A series of experiments was first carried out in order to determine the changes which occur in the phosphorus-content of pressed yeast and in the proportion of its unsaponifiable matter after the yeast has been incubated in the sugar solution and in the sugar-phosphate medium respectively.

A known weight of yeast was incubated for 24 hours in a litre of 4 % sugar solution and a similar quantity of yeast in a litre of the same solution to which 3.962 g.  $\text{NaH}_2\text{PO}_4$  and 0.286 g.  $\text{K}_2\text{HPO}_4$  had been added. A rapid current of oxygen was passed through both liquids; it is essential that the rate of oxygen current should be as nearly as possible the same in both experiments. The yeast was then filtered off and analysed. The easiest method of extracting the fat is to boil the yeast for two hours with normal hydrochloric acid and to extract the fat from the residue. The lipin is however decomposed by this method and it is therefore necessary to separate the fat from the yeast by repeatedly extracting the dried yeast with alcohol. It was generally found necessary to make from ten to twelve extractions of the yeast, using in each case from fifteen to twenty times its own weight of alcohol, the final extraction being carried out with boiling alcohol. The yeast was first treated with about twenty times its weight of 96 % alcohol to remove most of the water, the diluted alcohol filtered off, concentrated under diminished pressure to a small volume and the residue extracted with light petroleum. The alcohol from

subsequent extractions was evaporated under diminished pressure and the residues taken up in light petroleum.

In some of these experiments we determined the percentage of phosphorus in the fat obtained from each successive extract; no distinct regularity was observed: on the whole the phosphorus-content of the fat was higher in the earlier fractions and there was certainly no indication that the phosphorised fat was more firmly bound or more difficult to extract from the cell than the fat or other lipid constituents. Since lecithin and kephalin each contain about 4 % of phosphorus, the amount of phospholipin was calculated on this basis from the phosphorus-content of the fat.

The results are set out in the following table.

Table I.

No. of expt	Total lipid by alcohol extraction g.	Total P in yeast g.	Total P in lipid g.	% P in lipid g.	% N calc. as protein	% unsap. matter
<i>I. Analysis of 15 grams of original pressed yeast.</i>						
833	0.1736	0.083	0.0023	1.32	1.42	—
835	0.1554	0.096	0.0020	1.29	1.83	—
837	0.1378	0.097	0.0003	0.22	1.58	—
839	0.1729	0.088	0.0018	1.06	1.72	—
841	0.1552	0.085	0.0019	1.23	1.69	—
845	0.1637	0.074	0.0019	1.14	1.69	31.1
847	0.1702	0.104	0.0021	1.21	1.53	35.4
Mean values	0.1613	0.090	0.0018	1.07	1.64	33.2
<i>II. Analysis of similar amount of yeast after incubation in 4 % glucose solution.</i>						
833	0.3320	0.098	0.0043	1.45	1.23	—
835	0.5031	0.106	0.0026	0.51	1.58	—
837	0.3508	0.098	0.0028	0.82	1.58	—
839	0.5002	0.090	0.0044	0.87	1.58	—
841	0.4080	0.091	0.0043	1.04	1.53	—
843	0.3740	0.079	0.0034	0.94	1.61	32.3
845	0.5455	0.093	0.0053	0.97	1.52	27.3
Mean values	0.4305	0.094	0.0039	0.94	1.52	30.5
<i>III. Analysis of similar amount of yeast after incubation in 4 % glucose-phosphate solution.</i>						
833	0.8235	0.134	0.0067	0.81	1.26	—
835	0.9192	0.146	0.0076	0.80	1.49	—
837	0.7630	0.154	0.0059	0.78	1.42	—
839	0.9172	0.144	0.0060	0.65	1.56	—
841	0.8254	0.143	0.0070	0.85	1.61	—
843	0.5770	0.120	0.0043	0.74	1.61	36.2
845	0.7794	0.127	0.0041	0.53	1.48	28.4
Mean values	0.8008	0.138	0.0058	0.74	1.49	32.3

From the figures given in the above table it follows that 100 g. of the original pressed yeast contain 1.08 g. of ether-soluble substance and 0.29 g. phospholipin, the latter being calculated from the phosphorus-content. The proportion of lipin in most of the samples examined was appreciably higher than this but one sample differed from the others in having an extremely low phosphorus-content and brought down the average amount.

After incubation in glucose solution alone, 100 g. of yeast contained 2.87 g. of total lipid matter and 0.65 g. phospholipin. After incubation in the glucose-phosphate medium the ether-soluble substance constituted 5.34 % of the original weight of pressed yeast and contained 0.97 g. of phospholipin. The

total amount of ether-soluble substance had increased to five times its original amount and that of phospholipin to three times its original value. The variations in the percentage of lipin found in the incubated yeasts were considerably less than those in the original samples of pressed yeast taken for investigation.

The variations in the amount of unsaponifiable matter were not sufficient and the number of determinations made were too few for us to be able to attach any significance to them. In all cases approximately 30 % of the ether-soluble matter consisted of unsaponifiable substance. Since the total amount of the unsaponifiable matter increases in proportion to the amount of fat formed and is often five or six times that originally present, it must have been made by the yeast from carbohydrate, and the process is aided by the presence of phosphate in the medium.

*Preparation of ether-soluble material for the isolation of lipins.*

As shown above, the average content of lipin in 100 g. of pressed yeast is only 0.30 g.: this is however raised to approximately 1 g. if the yeast be first incubated in the well oxygenated glucose-phosphate medium. We determined therefore to increase the proportion of lipid substances in the yeast before extracting it with alcohol, by incubating it in a suitable medium. The long and tedious process of extracting a reasonable amount of starting material was thus expedited and a considerable saving of alcohol effected.

In one experiment 3 kg. of pressed yeast, obtained from liquid brewery yeast, well washed with water, were added to 30 gallons of a 4 % solution of cane sugar containing 0.369 g.  $\text{Na}_2\text{HPO}_4$  and 0.0286 g.  $\text{KH}_2\text{PO}_4$  %, and for 28 hours a rapid current of air was blown through the solution. The yeast was allowed to settle overnight and in the morning the solution was decanted, the yeast filtered through a cloth, rubbed up with alcohol and again filtered by suction. The pressed-out yeast was ground with one-quarter of its weight of sand and extracted five or six times with alcohol. In this way from 3 kg. of yeast about 100 g. of extract were obtained containing roughly 20 g. of lipin. Determination of the amount of fat in a sample of the same yeast after it had been hydrolysed, showed that it contained nearly 180 g. of lipid matter.

*Separation of phospholipins.*

The phospholipin fraction was separated by repeatedly precipitating with acetone the ether solution of the total lipid material, and was obtained as a white amorphous substance. Subsequently a further separation into lecithin and cephalin fractions was made (see p. 379).

Throughout all work with lipins it is advisable to keep all flasks, cylinders, etc., thoroughly flushed with nitrogen so that oxidation may, as far as possible, be prevented.

The N:P ratio of the lipin was nearly 1:1; the nitrogenous impurity which gives so much trouble in preparing lecithin from animal tissues does not seem to be present to any large extent.

Table II. *Analysis of lipin from yeast.*

	Theoretical figure for dioleyl-lecithin	Mixed lipin		CdCl <sub>2</sub> - lecithin fraction	Kephalin fraction
% P	3.81	3.59	3.585	3.096	3.02
% N	1.72	1.87	1.44	1.79	1.38
N : P	1 : 1	1.15 : 1	0.89 : 1	1.28 : 1	1.01 : 1

*Products of hydrolysis of the lipin.*

The lipin is conveniently hydrolysed by boiling with 10 % sulphuric acid for 6 to 8 hours under a reflux condenser in a slow stream of nitrogen.

(a) *Nature of bases.* The aqueous hydrolysis liquid, after extraction with ether to remove fatty acids, was analysed to determine the total nitrogen (by Kjeldahl's method) and the amino-nitrogen (by the micro-method of Van Slyke). The proportion of choline to amino-ethyl alcohol can thus be estimated and the lecithin-kephalin ratio calculated.

The lipin from pressed yeast contained a larger proportion of kephalin than lecithin, whereas in that from "incubated" yeast they were present in nearly equal amounts. Only a small amount of material was available after purification and it was therefore impossible to effect a complete separation of lecithin from kephalin.

The following table shows the total and amino-nitrogen in the hydrolysis liquids together with the percentage composition of the lipin.

Table III.

Lipin from pressed yeast			Lipin from incubated yeast			
Without separation			Without separation	"Kephalin"	"Lecithin"	
	(a)	(b)	(c)	(d)	(e)	(f)
Total N ...	0.1273 g.	0.0679 g.	0.0708 g.	0.1113 g.	0.0302 g.	0.0811 g.
Amino-N ...	0.1123 g.	0.0494 g.	0.0346 g.	0.0451 g.	0.0187 g.	0.0264 g.
Calculated						
% lecithin ...	27.3	11.8	51.15	59.5	37.96	67.5
% kephalin	72.7	88.2	48.85	40.5	62.04	32.5

Before its separation, the pressed yeast has been standing for some days in the wort and a considerable amount of autolysis has taken place. On the other hand, after the yeast has been incubated in the sugar-phosphate solution, from 80 to 90 % of the fatty matter has been freshly formed and this has only been standing in contact with the medium for a comparatively short time. In this freshly formed lipin it is interesting that the proportion of lecithin is greater than in the lipin derived from the pressed yeast. MacLean [1915] has shown that the proportion of lecithin present in the lipins extracted from the tissues is greater if the tissue is procured in as fresh a condition as possible and dried as quickly as possible. There seems some reason therefore to believe that both in yeast and in animal tissues autolytic changes may occur by which lecithin is converted into kephalin.



On the other hand yeast, when supplied with carbohydrate and phosphate but with no nitrogenous matter, has to use the substances stored in its own cells for its nitrogen supply and the relative amount of the two lipins formed may conceivably be influenced by this factor.

An experiment was carried out in which nitrogen was added to the medium in the form of ammonium sulphate. Unfortunately for our purpose the addition to the medium of a nitrogenous substance resulted in an increase of the total weight of yeast and in a diminution of the total weight of fat present. In this case, protein is made in preference to fat so that we can only investigate the case of increased fat formation when the nitrogen is obtained from substances originally present in the yeast cell.

(b) *Nature of acids.* The fatty acids from the hydrolysed lipin were extracted with purified ether immediately after cooling, and amounted to 50–60 % of the original material; the iodine value of an aliquot portion of the extract showed 60–70 % of the acids to be unsaturated.

The mixture was separated into the liquid unsaturated acid and the solid saturated acid fractions by the lead salt method as follows.

The acids were dissolved in a little alcohol, sufficient 2 % aqueous KOH was added to make the mixture neutral to phenolphthalein followed by 15 cc. of a hot 7 % lead acetate solution for every gram of fatty acid present. After cooling for some hours, the aqueous layer was poured off from the precipitated lead salts, which were washed with hot water and drained. These lead salts were then warmed with ether under a reflux until all the particles had been loosened from the sides of the flask, the whole cooled and filtered in an atmosphere of nitrogen, thereby giving ether-soluble and ether-insoluble fractions.

#### *Treatment of ether-soluble lead salt fraction.*

The ether-soluble lead salts were decomposed by shaking with dilute HCl and washing the ether solution with water till the washings were free from acid. After drying over sodium sulphate, aliquot portions were taken to determine the weight and iodine value of the acids present and for bromination.

The iodine value determined by Hübl's method was 90, corresponding with that of oleic acid.

The absence of acids of a greater degree of unsaturation, *i.e.* with more than one double bond, was confirmed by the bromination results.

The unsaturated fatty acids freed from moisture and ether were dissolved in sufficient pure carbon tetrachloride to give a 2 % solution and cooled to 0°. To this was added slowly at 0° a 2 % solution of bromine in carbon tetrachloride till the solution was just tinged red. After about 30 minutes an additional slight excess of the bromine solution was run in and the mixture allowed to stand overnight at 0°. The solvent and excess bromine were removed under reduced pressure at laboratory temperature and the residual oil treated with a small amount of dry light petroleum. In no case was there any trace of the precipitation of solid tetrabromide, and a portion of the bromination

product without further treatment was found to contain 35.5 % bromine, corresponding to dibromostearic acid, which contains 35.9 % bromine.

It was found essential to exclude moisture and to allow the bromine to act only in small concentration, otherwise substitution products were formed which caused considerable trouble in the earlier stages of the work. The presence of insoluble hexa- and octo-bromides in the bromination products was never observed although small traces of a white insoluble material containing 14 % Br were found on two occasions; these were not identified.

*Treatment of ether-insoluble lead salt fraction.*

The insoluble material was treated either with dilute acetic or hydrochloric acid and shaken with ether to extract the liberated fatty acids and the ether solution was washed with water till the washings were neutral. After drying the ether solution over sodium sulphate, aliquot portions were taken for total weight, iodine value, molecular weight and melting-point determination.

It is most important that the i.v. of the "saturated" fatty acids be determined as it is almost impossible to obtain a quantitative separation of saturated fatty acids from unsaturated acids by the lead salt method when dealing with a mixture of a small proportion of saturated with a large proportion of unsaturated acid. A knowledge of the i.v. enables the amount of unsaturated acid still mixed with the saturated acid to be calculated, since oleic acid is the only unsaturated acid found in the lipin.

The molecular weight of the saturated acid was determined after recrystallising the acid from dilute alcohol, dissolving in 96 % alcohol and titrating against *N*/10 sodium hydroxide using phenolphthalein as indicator. Values were found lying between 251 and 260. The recrystallised acid melted at 55–57°.

Palmitic acid melts at 63° and has a molecular weight of 256. Since, however, a specimen of solid acid gave an i.v. of 20, some oleic acid was present and would account for the lowered melting-point and high molecular weight.

*Examination of lecithin and kephalin fractions.*

Further separation of the lipin into two fractions—the lecithin fraction and the kephalin fraction—was carried out by treating the lipin with excess of absolute alcohol. The residue insoluble in alcohol, which constituted the kephalin fraction, was filtered off and the solution treated with excess of 1 % alcoholic cadmium chloride solution to precipitate the insoluble lecithin-cadmium chloride complex. The compound formed by this addition was completely soluble in ether, yet it still contained one-third of its total nitrogen in the amino-form, indicating that the separation from kephalin was incomplete. The kephalin fraction previously separated by its insolubility in alcohol contained only two-thirds of its nitrogen as amino-nitrogen and was therefore a mixture of kephalin and lecithin in the ratio of 2 : 1. (See Table III.)

The only acids identified from the hydrolytic products of both fractions were palmitic and oleic acids and no evidence of the existence of any other acid was obtained.

The proportion of oleic acid was however greater in the acids derived from kephalin than in those derived from lecithin.

Table IV. *The products of hydrolysis of lipin.*

	Total lipin	Lecithin fraction	Kephalin fraction
Fatty acid % ... ..	61.0	64.0	62.0
I.V. ... ..	—	64.95	72.45
Fatty acids of ether-soluble lead salts			
(i) Yield % ... ..	(a) 53.0 (b) 55.0	(a) 70.0 (b) 70.0	(a) 81.0 (b) 66.0
(ii) I.V. ... ..	(a) 89.97 (b) 87.73	(a) 75.9 (b) 75.9	(a) 68.3 (b) 83.4
(iii) Br in bromination product %	(a) 39.5 (b) 35.5	(a) 35.51 —	(b) 35.42 —
Theory for dibromostearic acid ...	35.9		
Fatty acids of ether-insoluble lead salts			
(i) Yield % ... ..	(b) 45.0	(a) 30.0	(b) 20
(ii) I.V. ... ..	(b) 41.0	54.96	61.9
(iii) Mol. wt. ... ..	(a) 251.4 (b) 263.0	260	259
(iv) M.P. ... ..	(a) 53° (b) 54.5°	60°	58°
Calculated from I.V. since oleic is the only unsaturated acid present			
Unsaturated % ... ..	(b) 73.6	72.0	74.5
Saturated % ... ..	26.4	28.0	25.5
	100.0	100.0	100.0

As shown in Table IV, the fatty acids of yeast lipins consist of a little palmitic and much oleic acid.

The isolation of the saturated and unsaturated acids in the above experiments was unsatisfactory. The lead salt method of separation is known, however, to work badly when the mixture to be separated contains a large excess of the unsaturated constituent.

The only satisfactory method is by the fractional distillation of the methyl esters but for this a larger quantity of material must be available than we had at our disposal.

Combining the information derived from a knowledge of the I.V. and the amino-N : total N ratio, it is possible to calculate the composition of the yeast lecithin and kephalin, thus:

From nitrogen figures

kephalin fraction contains 62 % kephalin  
38 % lecithin  
lecithin fraction contains 67.5 % lecithin  
32.5 % kephalin

I.V. of mixed fatty acids of

kephalin fraction: 72.45  
lecithin fraction: 64.95

Let  $x$  be i.v. of mixed fatty acids of kephalin

„  $y$  „ „ lecithin

Then  $62x + 38y = 7425$

$32.5x + 67.5y = 6495$

Hence  $x = 82.2$

$y = 56.55$

As only oleic and palmitic acids appear to be present

i.v. of kephalin acids is due to 91 % oleic acid

9 % palmitic acid

i.v. of lecithin acids is due to 62.6 % oleic acid

37.4 % palmitic acid

These results would suggest that yeast kephalin is a mixture of 82 % dioleyl-kephalin and 18 % oleyl-palmityl-kephalin, and the lecithin a mixture of 75 % oleyl-palmityl-lecithin and 25 % dioleyl-lecithin.

#### *Examination of the "acetone-soluble" fat.*

After separation of the lipin, the acetone solution was concentrated, reprecipitated with acetone and the filtered liquid again concentrated under reduced pressure at 40° till free from solvent. The residue consisted of the "acetone-soluble fat." It was a viscid, deep yellow oil which on standing in the cold room deposited white crystals of sterol or sterol ester.

Analysis of this fat showed the presence of only small amounts of phosphorus. The acid value remained fairly constant whereas the saponification value of fat from pressed and incubated yeast showed a distinct difference.

Table V. *Analysis of acetone-soluble fat from*

	(a) Pressed yeast				(b) Incubated yeast		
% P	0.091	0.062	0.048	0.345	0.252	0.075	0.114
A.V.		6.22			7.52	3.26	8.29
S.V.	172	175	176	172	122	128.8	137.2
I.V.		128.1				—	

#### *Hydrolysis of fat.*

After saponification of a sample of fat with alcoholic potash in a current of nitrogen, the alcohol was removed by heating on a water-bath and the residue dissolved in water. It was then extracted with ether to remove the unsaponifiable matter, acidified and again extracted to obtain the fatty acids.

(a) *Nature of acids.* As in the case of the lipin examination aliquot portions of the dry ether solution of the fatty acids were taken for iodine value, weight determination and the lead salts separation.

The lead salts soluble and insoluble in ether were separately decomposed as previously described and the liberated acids examined for iodine value, bromination products, molecular weight and melting point.

It was found that *the iodine value of the mixed acids was higher than that of the mixed lipin acids*, and on bromination, di- and tetra-bromo-derivatives were obtained which were separated by taking advantage of the sparing solubility of the tetrabromo-compound in light petroleum. It was found that the proportion of dibromide to tetrabromide was very variable.

Analysis showed that dibromostearic and tetrabromostearic acids were present, hence the existence of oleic and linolic acids in the original fat may be inferred. Palmitic acid was also isolated.

Table VI.

% fatty acid in fat	...	...	...	...	50.0-60.0 (Mean of 5) 55.1
I.V. of mixed fatty acids	...	...	...	...	72.75-88.3 (Mean of 6) 77.7
% total fatty acid present as unsaturated acid	...	...	...	...	50.0-56.0 (Mean of 4) 51.5
I.V. of mixed unsaturated acids	...	...	...	...	97.1-119.6 (Mean of 4) 107.8
% bromine in dibromo-acid	...	...	...	...	34.95 35.50 36.05
Theory for dibromostearic acid	...	...	...	...	35.9
% bromine in tetrabromo-acid	...	...	...	...	53.46
Theory for tetrabromostearic acid	...	...	...	...	53.33
% total fatty acid present as saturated fatty acid	...	...	...	...	48.0-50.0 (Mean of 2) 49.0
I.V. of saturated acids	...	...	...	...	24.0
M.P. of recrystallised acid	...	...	...	...	55° 57.5° 63°
M.P. of palmitic acid	...	...	...	...	63°
Molecular weight	...	...	...	...	249-258 (Mean of 3) 253.7
Theory for palmitic acid	...	...	...	...	256

(b) *The unsaponifiable fraction.* (See Table VII.) The unsaponifiable material was examined for the presence of ergosterol by warming with light petroleum and recrystallising the soluble material from alcohol. A beautiful white crystalline solid was obtained with an iodine value about 200; m.p. 140-145°. The substance crystallised in characteristic elongated hexagonal plates.

The free sterol in the fat was determined by dissolving 0.4 g. in 50 cc. of 95 % alcohol and adding 50 cc. of a hot 1 % solution of digitonin in 90 % alcohol. After standing a few hours the digitonin-sterol compound was filtered through a Gooch crucible and dried at 110° before weighing. The total sterol was estimated by first hydrolysing 0.4 g. of fat, extracting the unsaponifiable material and precipitating with digitonin as above.

It was found that the fat contained 1 % free ergosterol and some 14 % combined sterol, and that this sterol constitutes 50 % of the total unsaponifiable material.

Since the iodine value of the total unsaponifiable matter is approximately half that of the ergosterol, it follows that the remainder of the unsaponifiable

matter must consist almost entirely of saturated compounds, which we are now further investigating.

Table VII.

					Smedley-MacLean and Thomas [1920]	Smedley-MacLean and Daubney	
% unsaponifiable matter in fat					39.8	24.3-33.35	
						Mean of 6	29.6
I.V.	...	...	...	...	—	95.81	
% ergosterol in fat	...	...	...	...	17.23	15.63	
I.V.	...	...	...	...	177.1	188.5	200.1
M.P.	...	...	...	...	—	140-5°	140-5°
% free ergosterol	...	...	...	...	4.9	1.24	
% combined ergosterol	...	...	...	...	12.3	14.19	
% ergosterol in unsapon. material	...	...	...	...	43.3	52.7	

## SUMMARY AND DISCUSSION OF RESULTS.

(1) It has previously been shown that the effect of adding alkali phosphates to a well oxygenated solution of glucose in which yeast is incubated is approximately to double the total amount of ether-soluble (lipoid) substance. At the same time the proportion of lipin is somewhat diminished but the total quantity is much increased. The small amount of nitrogen necessary to form the new lipin must be derived from the nitrogenous material previously existing in the yeast. The phosphorus is presumably derived from the phosphate: the total amount of phosphorus in the yeast is very largely increased and evidence has already been furnished that the phosphate is taken up in association with carbohydrate. No significant variation in the proportion of unsaponifiable matter was observed.

It appears therefore that the addition of phosphate to a sugar solution increases the formation of fat, lipin and sterol and that, as in the animal organism, these lipid constituents maintain a fairly constant balance.

(2) The phospholipins of yeast consist of lecithin and kephalin and in both of these the same fatty acid radicals, oleyl and palmityl, appear to be present. The proportion of oleyl is apparently greater in the kephalin than in the lecithin molecule. There seems to be some evidence that the proportion of kephalin is higher in lipin from yeast that has undergone partial autolysis than in the freshly produced lipin—a result in accordance with the findings of MacLean for animal tissues. The saponification value (175) of the lipoid matter from the pressed yeast obtained after the yeast had stood for some time in contact with the wort was considerably higher than that (130) of fatty matter from the yeast which had been incubated in the glucose-phosphate medium.

(3) Perhaps the most interesting result established by the experiments now described is that the fatty acids which occur as constituents of the phospholipins of yeast have a lower iodine value than the fatty acids of the acetone-soluble fat. Few reliable data exist for comparing the degree of unsaturation of the acids from the lipins and the acetone-soluble fat respectively of the same organ. The acids of the liver were examined by Kennaway and

Leathes [1909] who found that though the highly unsaturated acids were not confined in the liver to the lipins, yet generally speaking the acids of the liver were more unsaturated than those of the simple glycerides. A similar relation was shown by Bloor [1926] to hold for the fatty acids of the heart. The iodine value of the acids from the heart lipins is very considerably higher than that of the acids from the non-phosphorised fat. Arachidonic acid, a twenty-carbon acid with four double bonds, constitutes 6 % of the lipin acids but only 2 % of the acids from the simple glycerides.

On the other hand, when Bloor [1924] examined the fatty acids which occur in the blood he found that, contrary to expectation, the more highly unsaturated acids for the most part did not occur in the lipins of the blood but were associated with the sterols.

Yeast fat is characterised by the very large proportion of unsaponifiable matter which it contains. This may constitute one-third by weight of the whole fat: approximately half of the unsaponifiable matter consists of the sterol characteristic of the fungi, ergosterol, and nearly the whole of this is in combination with fatty acids as esters. The remainder of the unsaponifiable matter consists of a saturated yellow oil which is being further investigated. In yeast it is the portion of fat containing steryl and glyceryl esters from which the linolic acid is derived. In the yeast lipins oleic acid seems to be the only unsaturated acid present.

(4) Another point of interest about the yeast lipins is that they occur in a less complicated mixture than is usually found in other tissues. In both the kephalin and lecithin fractions from yeast, oleic and palmitic acids appear to be the only two acids present and of these oleic acid is present in very much larger amount. We may conclude therefore that dioleyl- and palmityl-oleyl lecithins and kephalins occur in yeast and that the dioleyl compounds are present in the greater amount. The proportion of the oleic acid is somewhat greater in the kephalin than in the lecithin fraction. In the acetone-soluble fat, on the other hand, saturated and unsaturated acids seem to be present in approximately equal proportions. Very little information is available as to the nature of the acids present in plant lipins. The lipins of the Soya bean were investigated by Levene and Rolf [1925, 1, 2; 1926] who showed that the iodine value of the mixed lipin acids was low compared with that of the mixed lipin acids from animal tissues: the percentage of saturated acid was also exceptionally low. The unsaturated acids isolated from the lipins all contained eighteen carbon atoms and comprised oleic, linolic and linolenic acids. The saturated acids accounted for 15 %, the unsaturated for 43 % and the acids constituting the remaining 42 % were not satisfactorily identified, though some evidence was obtained of the existence of an unsaturated hydroxy-acid. In the work of other observers on various vegetable lipins, oleic acid appears to have been the only acid identified. There are no data available from which a comparison of the acids from the lipins and acetone-soluble fat of the same plant can be made.

On the whole, from such evidence as is available, it seems that the acids of the vegetable lipins have a lower degree of unsaturation than the acids derived from the animal lipins and they are also characterised by a low proportion of saturated acid. In some cases at any rate unidentified acids of unknown structure appear to be present.

Arachidonic acid has not so far been shown to occur in the lipins of the plant kingdom, linolenic acid being the most unsaturated acid yet isolated. Further data as to the nature of the acids present in plant lipins are much needed; a comparison of the acids which occur in the animal and plant lipins may possibly help us to understand something of the functions of the lipins themselves.

Certainly in the yeast lipins, the radicals of highly unsaturated acids seem to be absent and the doubly unsaturated acid of yeast fat occurs only in combination with sterol or glycerol.

We desire to express our indebtedness to the Food Investigation Board of the Department of Scientific and Industrial Research for grants which have enabled us to carry out this work: our thanks are also due to Miss E. Clenshaw for her assistance in carrying out part of this work and to the Directors of Messrs Watney, Coombe, Reid and Co. for supplying the yeast for this investigation.

## REFERENCES.

- Austin (1924). *J. Biol. Chem.* **59**, Proc. lii.  
Bloor (1924). *J. Biol. Chem.* **59**, 543.  
—— (1926). *J. Biol. Chem.* **68**, 33.  
Hinsberg and Roos (1904). *Z. physiol. Chem.* **42**, 189.  
Hoppe-Seyler (1866). *Med.-Chem. Untersuch.* **1**, 142.  
—— (1879). *Z. physiol. Chem.* **2**, 427; **3**, 374.  
Kennaway and Leathes (1906). *Proc. Roy. Soc. Med.*, Feb.  
Koch (1902). *Z. physiol. Chem.* **37**, 181.  
Levene and Rolf (1925, 1). *J. Biol. Chem.* **62**, 759.  
—— (1925, 2). *J. Biol. Chem.* **65**, 545.  
—— (1926). *J. Biol. Chem.* **68**, 285.  
MacLean (1915). *Biochem. J.* **9**, 351.  
Neville (1913). *Biochem. J.* **7**, 347.  
Sedlmayer (1903). *Z. ges. Brauwesen*, **26**, 381.  
Smedley-MacLean and Hoffert (1923). *Biochem. J.* **17**, 720.  
—— (1924). *Biochem. J.* **18**, 1273.  
Smedley-MacLean and Thomas (1920). *Biochem. J.* **14**, 483.



# LV. NOTE ON SOME STEROL COLOUR RE-ACTIONS IN THEIR RELATION TO VITAMIN A.

By OTTO ROSENHEIM.

*From the National Institute for Medical Research, Hampstead, N.W. 3.*

*(Received February 26th, 1927.)*

THE discovery of the brilliant blue colour reaction of cod-liver oil with  $\text{AsCl}_3$  and its suggested association with both vitamin A and cholesterol [Rosenheim and Drummond, 1925] led to a search for a similar reaction, obtainable from sterols or their derivatives under conditions which would throw light on the nature of the chromogen concerned in the cod-liver oil reaction. Starting from the assumption that oxidative processes are connected with the formation of the blue pigment in sterol reactions, the action of mild oxidising agents on sterols was investigated. It was found that treatment of cholesterol with benzoyl peroxide in chloroform solution produced a chromogenic substance which gave with  $\text{AsCl}_3$  a blue colour indistinguishable in appearance from that given by cod-liver oil with  $\text{AsCl}_3$ . The possibility of the formation of an aldehydic sterol derivative on mild oxidation suggested further a study of the influence of formaldehyde on the colour reactions of sterols. Again a change from the usual red colour to a clear blue was found to be produced when  $\text{AsCl}_3$  reacted with cholesterol in the presence of nascent formaldehyde. This reaction is conveniently carried out by adding  $\text{AsCl}_3$  to a solution of cholesterol in methylal. In this case, however, the chromogen cannot be isolated, as it is slowly converted into the pigment in the course of the reaction<sup>1</sup>.

Although the blue pigment formed from cholesterol resembles on superficial inspection that obtained in the cod-liver oil reaction, a more detailed investigation raised doubts as to their identity. This doubt was increased when it was found that cholic acid also gives rise to a similar blue colour reaction after heating with benzoyl peroxide in chloroform solution. The latter reaction again demonstrates the near relationship of the bile acids to cholesterol, the nature of which has been cleared up by the work of Windaus and Neukirchen [1919]. The similarity of these colour reactions to that of the chromogen of cod-liver oil suggests that there exists a similarity between the carbon ring

<sup>1</sup> Antimony trichloride, trichloroacetic acid, dimethylsulphate, etc., may be used as condensing agents in place of  $\text{AsCl}_3$  in all these reactions under slightly modified conditions. The various phytosterols and ergosterol react in a similar way. In view of the multiplicity of sterol reactions already available, a detailed description of the new reactions may be dispensed with.

system of the sterol molecule and that of the unknown chromogen and lends support to the view that oxidative changes of the sterol molecule may be concerned in the vitamin A formation from sterols.

Evidence is accumulating indicating that the blue colour reaction of cod-liver oil is specific for vitamin A [Rosenheim and Webster, 1926, 1927, 1]. As the new colour reactions of sterols are of interest only in their relation to this vitamin, a short description of one of them is sufficient in this respect. The chromogen is rapidly formed on evaporating to dryness a chloroform solution of cholesterol and benzoyl peroxide in equimolecular proportions. A quantitative colorimetric test in Lovibond's colorimeter showed that the maximum colour production is reached after boiling the solution for about two hours (1 mg. of the reaction product + 2 cc.  $\text{SbCl}_3$  solution equals 12 blue units). All attempts to isolate the chromogen in a pure condition from the complex mixture of amorphous oxidation products have so far failed.

The properties of the resinous product obtained resemble those described for a similar mixture called "oxycholesterol" by Lifschütz, and like the latter material it can also conveniently be prepared from cholesterol dibromide by debromination with sodium acetate in alcoholic solution [Lifschütz, 1919]. The substance to which Lifschütz ascribes, without justification, the formula  $\text{C}_{27}\text{H}_{46}\text{O}_2$ , is undoubtedly a complex mixture [see Windaus, 1908; Windaus and Lüders, 1921; Gardner, 1921], one constituent of which gives the green colour reaction, considered as characteristic for "oxycholesterol" by Lifschütz. The material also gives a blue reaction with  $\text{AsCl}_3$ <sup>1</sup>, but it appears doubtful whether the constituent giving rise to the green Lifschütz reaction is identical with the chromogen of the blue pigment. In any case there can be no doubt that Lifschütz's "oxycholesterol" has no bearing on the blue cod-liver oil reaction with  $\text{AsCl}_3$ , since, according to Lifschütz's and my own observations, the typical green colour reaction is not given by any liver fat (nor by cod-liver oil).

On the other hand, the fact that one constituent of the oxidation products of cholesterol gives a blue colour with  $\text{AsCl}_3$  suggested an investigation of its behaviour when added to such vegetable or animal oils as do not react with  $\text{AsCl}_3$  by themselves. It was found that in every case examined the production of the blue colour was completely inhibited when a purified preparation (from cholesterol dibromide) was dissolved in any natural oil or fat. This instability of the artificial chromogen in solution in a natural oil excludes its identity with the chromogen of cod-liver oil. The rapid destruction of the artificial chromogen appears to be connected with the presence of unsaturated linkages in the natural oils, for a freshly prepared solution in a neutral solvent, such as liquid paraffin, gives an intense positive reaction with  $\text{AsCl}_3$ . Even in this case, however, the chromogen gradually disappears on keeping under laboratory conditions.

<sup>1</sup> In the course of an examination of the sterols of echinoderms [Page, 1923], and of *Boletus granulatus* [Marston, 1924], the same observation is recorded incidentally.

Although in its relative instability the artificial chromogen resembles the chromogen of cod-liver oil, it may be pointed out that the blue pigment produced by  $\text{AsCl}_3$  from the cod-liver oil chromogen is typically unstable, the colour fading within a few minutes, whilst the blue colour given by the artificial product, when once formed, remains unchanged for many days. A characteristic difference in the absorption spectrum of the two pigments may be adduced as a further reason against their identity.

A few feeding experiments with the mixture of substances obtained on oxidation of cholesterol were kindly carried out by my colleague, Mr Webster. The results so far obtained, although not yet completely conclusive on account of the instability and impurity of the material, are against the view of its having the properties of vitamin A. In consideration of the relationship which has recently been shown to exist between ergosterol and the antirachitic vitamin D [Rosenheim and Webster, 1927, 2; Windaus and Hess, 1927], we are at present investigating the possible relation of vitamin A to the oxidation products of ergosterol.

#### REFERENCES.

- Gardner (1921). *Biochem. J.* **15**, 244.  
Lifschütz (1919). *Z. physiol. Chem.* **106**, 271.  
Marston (1924). *Austral. J. Exp. Biol.* **1**, 53.  
Page (1923). *J. Biol. Chem.* **57**, 471.  
Rosenheim and Drummond (1925). *Biochem. J.* **19**, 753.  
Rosenheim and Webster (1926). *Lancet*, ii, 806.  
—— — (1927, 1). *Biochem. J.* **21**, 111.  
—— — (1927, 2). *Lancet*, i, 306.  
Windaus (1908). *Arch. Pharm.* **246**, 117.  
Windaus and Hess (1927). *Nachr. Ges. Wiss. Göttingen*, 175: *Proc. Soc. Exp. Biol. Med.* **24**, 461.  
Windaus and Lüders (1921). *Z. physiol. Chem.* **115**, 257.  
Windaus and Neukirchen (1919). *Ber. deutsch. chem. Ges.* **52**, 1915.

## LVI. THE PARENT SUBSTANCE OF VITAMIN D.

BY OTTO ROSENHEIM AND THOMAS ARTHUR WEBSTER.

*From the National Institute for Medical Research, Hampstead, N.W. 3.*

*(Received April 4th, 1927.)*

Two years ago we found that cholesterol, having the characteristics then known of the pure substance, was rendered antirachitic by exposure to ultra-violet light [Rosenheim and Webster, 1925]. This fact was also discovered independently, and at about the same time, by the American observers Steenbock and Black [1925] and Hess, Weinstock and Helman [1925].

Although this discovery has since been confirmed and accepted by numerous investigators in many countries, and has found practical application for the cure of rickets in children, we were able to show last year [Rosenheim and Webster, 1926, 1] that cholesterol when purified still further by a chemical method had lost the property of becoming antirachitic by irradiation. In agreement with this observation, it was found that chemically purified cholesterol no longer possesses the absorption spectrum in the ultra-violet region which is characteristic for cholesterol purified by physical means only.

These facts threw new light on the photo-chemical formation of vitamin D. It became evident that the precursor of vitamin D (= provitamin) is not cholesterol itself, but an unknown substance associated with cholesterol as obtained from all natural sources (gallstones, brain, eggs, etc.)<sup>1</sup>.

The fact that the presence of an impurity in such a well crystallised substance as cholesterol has for more than a hundred years escaped recognition by chemists is explained by the minute amount (1:2000) concerned, and emphasises the delicacy of the biological test. It may be pointed out that the same method which enabled Reinitzer [1888] to arrive at the correct empirical formula of cholesterol, i.e. bromination, has now led also to the discovery of the biologically pure cholesterol.

The newly established facts proved valuable for the rapid progress of the work on the nature of the provitamin, in so far as they enabled us to rely, to a large extent, on the spectroscopic test in place of the tedious animal experiment. Although such a test had already been tentatively suggested by Schlutz and Morse [1925], a firm basis for its application was only established when we showed by animal experiments that the absence of absorption bands

<sup>1</sup> From the historical point of view, it is interesting to note that the suggestion of an impurity in cholesterol as the causative factor concerned in its activation by ultra-violet light had already been considered by Steenbock and Black [1925] and by Schlutz and Morse [1925], the latter observers basing themselves on spectrographic observations only.

in the ultra-violet coincided with the loss by cholesterol of its property of becoming antirachitic on irradiation. It is evident that the destruction or absence of provitamin can be rapidly proved by a negative spectroscopic test, whilst the animal experiment remains indispensable in confirmation of positive spectroscopic evidence.

In the search for the nature of the provitamin we had the privilege of collaborating with Prof. Windaus (Göttingen). Separate preliminary communications on this part of the work, the outcome of mutual suggestions, were made simultaneously by Prof. Windaus [Windaus and Hess, 1927], and by ourselves [Rosenheim and Webster, 1927, 1], according to a friendly arrangement.

In the first instance we established the fact that not only cholesterol but the vegetable sterols also could be freed from the provitamin by bromination. It was found that stigmasterol ( $C_{30}H_{48}O$ ), containing two double bonds, and sitosterol ( $C_{27}H_{46}O$ ), with one double linkage, when purified by way of the bromides, also yield no vitamin on irradiation; whereas all vegetable oils, and the sterols obtained therefrom and not so purified, readily acquire antirachitic properties under the action of ultra-violet rays.

Ordinary cholesterol may be freed from provitamin, as we have since discovered, not only by bromine, but also by various other chemical as well as physical methods. Thus pure cholesterol may be prepared from cholesteryl chloride, which cannot be activated itself, by conversion into cholesterol acetate [Mauthner and Suida, 1894] and subsequent saponification. Treatment of cholesterol in acetone solution by potassium permanganate, whilst leaving cholesterol unattacked, removes the provitamin by oxidation. Of the physical methods the simplest consists in over-irradiation and subsequent recrystallisation. Further, repeated treatment with an efficient charcoal frees ordinary cholesterol from provitamin, but whether this is due to adsorption or oxidation has not yet been investigated.

Whilst these results afforded no means for the isolation or even concentration of provitamin, more direct evidence as to its nature was furnished by the observation that, unlike the vitamin obtained from it, provitamin forms an addition compound with digitonin. Although the product recovered from the digitonide of ordinary cholesterol by fractional extraction with xylene contains provitamin in increased amount, the complete separation of the latter from cholesterol or other sterols by this method does not appear to be practicable.

These observations afford significant information with regard to the chemical nature of provitamin. We may conclude that it is destroyed by oxidation and by bromine, and that it possesses the properties of a sterol, in so far as it forms an addition product with digitonin. The sensitiveness of provitamin to oxidative processes is a characteristic of only one of the known sterols, *i.e.* the highly unsaturated ergosterol ( $C_{27}H_{42}O$ ). Its discoverer, Tanret [1890, 1908] had already studied the oxidation of ergosterol under the influence

of light and air, as evidenced by its change in colour, fall of melting point and optical rotation [see also Schulze and Winterstein, 1906]. Two other properties of provitamin are characteristic also for ergosterol, namely its destruction by bromine<sup>1</sup> and the formation of an insoluble digitonide [Smedley-MacLean and Thomas, 1920].

This similarity in their known properties suggested to Prof. Windaus that provitamin may be nearly related to, if not identical with, ergosterol and we therefore took up again, at his suggestion, the study of ergosterol which we had previously found [Rosenheim and Webster, 1926, 2], after irradiation "to be highly protective even in doses of one milligram" when comparing it under identical conditions with 5 mg. doses of irradiated cholesterol on the same batch of rats.

In view of the more recent work on the absorption spectrum of cholesterol by Heilbron, Kamm and Morton [1926] and by Pohl [1926], we compared the absorption spectrum of ergosterol with that of a specimen of ordinary cholesterol. One would expect ergosterol, with its three double bonds, to show a greater absorption in the ultra-violet region than cholesterol, which has only one double linkage. It was, indeed, found that ergosterol exhibited a very pronounced absorption in the ultra-violet region, the characteristic bands of absorption being identical with those of cholesterol but of enormously greater intensity. The intensity of the absorption was estimated to be 1500-2000 times as strong as that of brain cholesterol<sup>2</sup>. As the result of the irradiation the absorption bands disappear just as in the case of cholesterol; at the same time the product loses its property of being precipitated by digitonin. If provitamin were ergosterol, or an unsaturated sterol of similar constitution, the amount present in ordinary cholesterol would be of the order of 0.05 %, or one part in 2000, and irradiated ergosterol should in this case possess antirachitic activity in correspondingly small doses.

These assumptions have been fully confirmed by biological tests on rats. A daily dose of 1/10000 mg. of irradiated ergosterol cured and prevented rickets in rats kept on a rachitogenic diet. Tests with still smaller quantities, not yet completed, indicate that the limit of activity will prove to be less than 1/50000 mg. Irradiated ergosterol is therefore the most potent antirachitic substance known, 5 mg. being equivalent to approximately 1 litre of a good cod-liver oil.

It is of interest to note that a dose 10000 times greater than what is at present regarded as an effective dose, produced no obvious ill effects on rats under the experimental conditions used.

These results seem to justify the conclusion that the naturally occurring

<sup>1</sup> See also Rosenthal [1922]. According to previous unpublished observations of Windaus, ergosterol cannot be recovered unchanged from its amorphous bromination products.

<sup>2</sup> Since the provitamin content of cholesterol, purified by physical methods, is variable (see later) this figure necessarily varies with different specimens and may be as high as 1:10,000 or more.

parent substance of vitamin D is ergosterol or a sterol possessing the same absorption spectrum and physiological activity. The admixture in cholesterol from all animal sources (brain, eggs, gallstones, etc.) of a sterol with more than one double bond seems likely, in view of the well-known occurrence in phytosterols from vegetable oils, seeds, etc., of other representatives of this class, containing two double bonds. In this connection the recent work of Anderson [1927] is of interest; he drew attention to the fact that cholesterol preparations obtained from different sources show differences in physical properties, which are ascribed to the probable admixture of isomeric or different sterols. Their partial separation from cholesterol should be possible not only by fractional distillation in a high vacuum, as found by Windaus, but also by fractionation in superheated steam [see the suggestive results of Gardner, 1921].

It should be added that although ergosterol was originally isolated by Tanret from ergot, and named accordingly, similar or identical sterols ("mycosterols") have been obtained from a wide range of the lower plants, occurring, for example, abundantly in the fat extracted from certain yeasts [Smedley-MacLean and Thomas, 1920; Windaus and Grosskopf, 1922]. The ergosterol employed in our own experiments above quoted was actually prepared from ergot, whilst that used by Prof. Windaus was obtained from yeast.

The action of ultra-violet light on ergosterol leads to an obvious physical change and the production of a yellowish resin. The nature of the intramolecular change which gives rise to the vitamin formation is at present unknown.

### *Experimental.*

*Methods.* The irradiation of the substances to be tested, and the feeding experiments, were carried out by the technique described in our former communications. Negative controls with the basal diet only, as well as positive controls (basal diet + 0.1 cc. cod-liver oil), were made in every set of experiments, although not specifically reported in the tables. For the diagnosis of rickets we relied on the evidence of the radiograph and the estimation of the content of inorganic phosphates in the blood. The large number of radiographs necessary for this work were again kindly taken for us in the department of our colleague, Mr J. E. Barnard, F.R.S., to whom we wish to express our best thanks. It might be mentioned that the results were in all cases decisive, the rats showing either severe rickets or complete absence of rickets.

#### *I. Removal of the provitamin from various sterols.*

(1) *Phytosterols.* The method originally worked out for the separation of stigmasterol from other phytosterols by Windaus and Hauth [1906] depends on the insolubility of the tetrabromide of stigmasterol acetate. The necessity of using bromine in its preparation explains, in the light of our present knowledge, the previously surprising result that this naturally occurring sterol could not be activated. It may be pointed out that even the presence of two unsaturated linkages in a sterol is apparently not sufficient to enable it to

act as a provitamin. Stigmasterol (M.P. 170°) irradiated in air for one hour and administered in daily doses of 2 mg. and 4 mg. was unable to protect rats against rickets (see Table I).

Table I.

Substance	Stigmasterol	Sitosterol	Cholesterol recovered from over-irradiated ordinary cholesterol	Olive oil; fraction of "unsaponifiable" not precipitated by digitonin	Cholesterol from ordinary cholesterol-digitonide	
					Fraction (1)	Fraction (2)
Time of irradiation	1 hr.	1 hr.	30 min.	30 min.	30 min.	30 min.
Dose	2 mg.	2 mg.	2 mg.	40 mm. <sup>3</sup>	2 mg.	2 mg.
	4 mg.	4 mg.	4 mg.	40 mm. <sup>3</sup>	4 mg.	4 mg.
				160 mm. <sup>3</sup>		
Radiographic result	Rickets	Rickets	Rickets	Rickets	Normal	Normal

The dibromide of sitosterol acetate is soluble and remains in the filtrate from the stigmasterol derivative. Sitosterol itself may be prepared, without previous isolation of its bromide, from the above filtrate by the method described by Windaus and Hauth [1907].

The specimen of sitosterol (M.P. 136–137°) used in the feeding test was prepared from the phytosterols of Soja beans by the above method and when tested biologically in the same way as stigmasterol was found to be inactive (see Table I). Both sterols showed absence of absorption bands in the spectroscopic test.

(2) *Cholesterol*. The bromination method has already been described in our former communication [1927, 2].

(a) *Thionyl chloride* (or phosphorus pentachloride) may be used instead of bromine for the removal of provitamin. Cholesteryl chloride is obtained in the first instance and is subsequently converted into the acetate by boiling with zinc dust in acetic acid solution [Mauthner and Suida, 1894]. Cholesterol was recovered by saponification of the acetate, and gave a negative spectroscopic test.

It may be mentioned in explanation of this result that ergosterol is apparently destroyed by  $\text{PCl}_5$  [Reindel, Walter and Rauch, 1927].

(b) *Over-irradiation* destroys vitamin D, as has been previously shown. By converting all the provitamin present in cholesterol into vitamin D by irradiation, and subsequently destroying it by over-irradiation, it is possible to recover pure cholesterol after removal of the reaction products by recrystallisation. From an experiment devised to ascertain the possibility of reactivating cholesterol which had been deactivated by over-irradiation in the dry state, Hess, Weinstock and Sherman [1925] drew the conclusion that a substance "quite different from the original cholesterol had been formed." We found it necessary to irradiate a solution, and not the crystals, of ordinary cholesterol in order to induce the complete destruction of both the provitamin and the vitamin.



The substance was dissolved in ether and irradiated in a quartz flask for 10 hours. After evaporation of the ether, the cholesterol was recrystallised from alcohol and the whole process repeated. The final product was again recrystallised, irradiated for 30 minutes, and administered to rats in daily doses of 2 mg. and 4 mg. (see Table I). The spectroscopic, as well as the biological test, gave negative results.

(c) *Charcoal* easily removes the provitamin from ordinary cholesterol. Five g. cholesterol were dissolved in 50 cc. 96 % alcohol and boiled with 1 g. norit for half an hour, under reflux. The filtrate was treated again with 1 g. norit. The recovered cholesterol was found to be free from provitamin by the spectroscopic test (see Fig. 1, Plate I). The same result was obtained by using Merck's blood charcoal.

In view of the variable content in provitamin of commercial samples of cholesterol, possibly purified by means of charcoal, caution is indicated against the indiscriminate use of irradiated cholesterol as a source of vitamin D, when testing for the presence of vitamin A. We experienced a failure, unexplained at the time, of a series of feeding experiments dealing with vitamin A estimations owing to the employment of a cholesterol prepared from cod-liver oil by ourselves. The specimen in question had been carefully purified by charcoal in order to adsorb the persistent yellow pigments, which could not be removed by recrystallisation alone. We have since come across two commercial samples of cholesterol equally deficient in provitamin. We propose to use a solution of irradiated ergosterol in future work on vitamin A.

(d) *Potassium permanganate* in acetone solution may also be used. The procedure need not be described in detail, and it is sufficient to state that cholesterol obtained on concentration of the solution (after the filtration from  $\text{MnO}_2$ ) is spectroscopically inert.

## II. *Precipitation of provitamin by digitonin.*

We obtained decisive evidence for the formation of a digitonide of provitamin in the first instance by an indirect route. Olive oil (200 cc.) was saponified in the usual way. The unsaponifiable fraction could easily be activated by means of irradiation, as shown by a separate experiment. The alcoholic solution of the unsaponifiable fraction (0.64 g.) was precipitated with excess of digitonin. The filtrate from the digitonide, after evaporation *in vacuo*, was taken up in water and extracted with ether. The ether-residue was irradiated for 30 minutes, dissolved in 3 cc. liquid paraffin and administered in daily doses of 40 mm.<sup>3</sup> and 160 mm.<sup>3</sup> These amounts, corresponding to 2.5 cc. and 10 cc. of irradiated olive oil, were unable to prevent severe rickets, thus proving that provitamin had been completely removed by digitonin (see Table I).

Direct evidence of the presence of provitamin digitonide in ordinary cholesterol digitonide was furnished by precipitating ordinary cholesterol (from gallstones) with digitonin. The digitonide was recrystallised from

pyridine-alcohol, washed with ether and dried. The product was fractionally extracted with boiling xylene, fraction (1) representing cholesterol obtained after the first eight hours' extraction. The extraction was continued for another eight hours and the product labelled fraction (2). Spectroscopic examination of fraction (1) showed a considerable increase of absorption in the ultra-violet, indicating about double that of the original cholesterol. The animal experiment proved that both fractions prevented rickets after irradiation, when administered in daily doses of 2 mg. and 4 mg. (see Table I). No attempt was made to assess the difference in antirachitic activity of the two fractions by the animal experiment.

### III. *Ergosterol as provitamin D.*

The specimen of ergosterol used in this work was part of the same preparation, kindly put at our disposal by Dr H. A. D. Jowett, which we had previously found to be easily activated [1926, 2]. It had been prepared from ergot and since it possessed the constants given by Tanret [1908] for pure ergosterol, M.P.  $162^{\circ}$ ,  $[\alpha]_{5461}^{19^{\circ}} = -127'$ , must be considered as free from fungisterol.

(1) *Spectroscopic evidence.* The absorption spectra were photographically recorded by means of a Hilger's quartz spectrograph, using as a source of light a quartz mercury vapour lamp. An all-quartz cell of 10 mm. width was used, pure ether (free from peroxides) serving as the solvent.

The absorption of ultra-violet light by ergosterol was found to be enormous, even a solution of 1 : 2000 absorbing the whole of the region from  $310\mu\mu$  onwards (see 5, Fig. 1, Plate I). Many photographs were taken in which the absorption spectra of a series of dilutions were recorded, the exposure being kept constant. In Fig. 2 (Plate I) the strength of the solutions varied from 1 : 5000 (1) to 1 : 50000 (10), even this extreme dilution still showing distinct absorption between  $280-310\mu\mu$ . It is interesting to note that the absorption extends well into the ultra-violet region of sunlight, which is able to activate ordinary cholesterol, as we have previously shown.

In Fig. 1 (2) is shown for comparison the absorption spectrum of a solution 1 : 10 of ordinary cholesterol. The removal of the provitamin by charcoal treatment is illustrated by Fig. 1 (3), whilst (4) brings out the striking influence of the addition of ergosterol 1 : 20000, to a solution of pure cholesterol 1 : 10. By comparing (2) and (4) it will be seen that the absorption in the critical region of from  $280-310\mu\mu$  is almost identical, and the conclusion may be drawn that the absorption in the ultra-violet of ergosterol is about 2000 times that of ordinary cholesterol. This merely qualitative judgment was confirmed by quantitative measurements made in Göttingen (see footnote, p. 391).

Although the absorption of pure cholesterol in the ultra-violet is powerfully affected by the addition of this minute amount of ergosterol, it might be mentioned that no change in physical properties, such as melting point and

specific rotatory power which usually serve to the chemist as a criterion of purity, can be demonstrated in an artificial mixture of pure cholesterol containing 0.05 % ergosterol. The admixture of ergosterol cannot be detected by the usual colour reactions, since no specific colour reaction for it in the presence of other sterols is so far known.

The gradual disappearance of the characteristic absorption bands, which takes place on irradiation, may be taken to coincide with the formation of vitamin D, which is also brought into evidence by the coincident decrease of digitonin-precipitability. By taking a series of absorption spectra after exposing a 1 : 1000 ergosterol solution to the prolonged action of ultra-violet light, it was found that an exposure of approximately five hours was necessary to cause the complete "bleaching out" of the absorption. After the same period the product did not possess the property of forming an insoluble digitonide.

(2) *Biological evidence.* Two main series of experiments were carried out. In the first, ergosterol was irradiated as a solid in air for 30 minutes, and in the second series a solution in pure ether was irradiated. A quartz cell ( $10 \times 25 \times 25$  mm.) with a narrow tubular neck was employed in order to restrict the exposure of the solution to air as much as possible. The ether solution was irradiated in the closed cell for 30 minutes and 5 hours respectively. After evaporation of the solvent, the irradiated material was dissolved in olive oil, which had previously been tested for the absence of vitamin D by the animal experiment. The daily doses varied from 0.025 mg. to 0.0001 mg. ( $= 1/40$  to  $1/10000$  mg.). The results were identical in all cases, *i.e.* complete protection and cure were effected even by the smallest daily dose of  $1/10000$  mg. (see Table II). Experiments are in progress with still smaller doses. We have reason to believe that the limiting dose will be considerably less than  $1/50000$  mg. when the irradiation is carried out under the most favourable conditions in the absence of oxygen.

Table II.

Substance	Ergosterol (in ethereal solution)		Ergosterol (as a solid)
	30 minutes	5 hours	
Time of irradiation	30 minutes	5 hours	30 minutes
Dose in mg.	1/40, 1/80, 1/500, 1/1000 1/10000	1/40, 1/80, 1/500, 1/1000 1/10000	1/40, 1/80, 1/125, 1/250, 1/500, 1/1000 1/10000
Radiographic result	Normal	Normal	Normal

An interesting proof of the stability of irradiated ergosterol is afforded by the fact that a solution in an inactive oil kept for  $1\frac{1}{2}$  years was found to be still active when tested in daily doses of  $1/1000$  mg. This solution had served for our original work on the activation of ergosterol and had been kept in a closed flask in a cupboard.



Fig. 1.

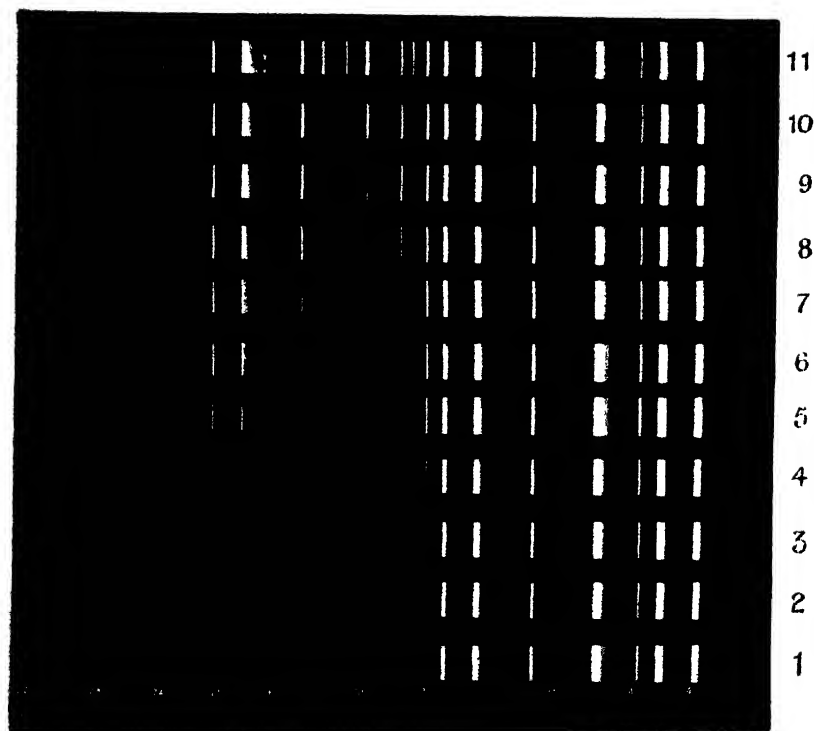


Fig. 2.



## SUMMARY.

(1) Cholesterol and phytosterols, purified by the methods described, can no longer be rendered antirachitic by ultra-violet light.

(2) The nature of the impurity present in ordinary sterols which is responsible for their activation by ultra-violet light has been investigated. The evidence obtained leads to the view that the impurity is a sterol of an unsaturated and labile type, of which ergosterol is the only known representative.

(3) Ergosterol was found to possess the same characteristic absorption spectrum in the ultra-violet as non-purified cholesterol, the intensity of the absorption, however, being enormously increased. Assuming it to be ergosterol, the amount of impurity present in ordinary cholesterol was judged by comparative spectroscopic examination to be of the order of 1 : 2000.

(4) Irradiated ergosterol in daily doses of 1/10000 mg. cures and prevents rickets in rats on a rachitogenic diet.

(5) It is concluded that the natural parent substance of vitamin D is ergosterol, or a highly unsaturated sterol of similar constitution, which is converted into vitamin D by irradiation.

## EXPLANATION OF PLATE I

Fig. 1. Comparative absorption spectra of cholesterol and ergosterol in solution in ether.

(1) Ether. (2) Ord. cholesterol in ether, 1 : 10. (3) Same after charcoal treatment. (4) Pure cholesterol, 1 : 10, plus ergosterol 1 : 20000. (5) Ergosterol, 1 : 2000.

Fig. 2. Absorption spectra of ergosterol.

(1-10) Ergosterol in ether, 1 : 5000 to 1 : 50000. (11) Ether.

Source of light: quartz mercury lamp.

Width of cell: 10 mm.

## REFERENCES.

- Anderson (1927). *J. Biol. Chem.* **71**, 407.  
 Gardner (1921). *Biochem. J.* **15**, 244.  
 Heilbron, Kamm and Morton (1926). *J. Soc. Chem. Ind.* **45**, 932.  
 Hess, Weinstock and Helman (1925). *J. Biol. Chem.* **63**, 305.  
 Hess, Weinstock and Sherman (1925). *J. Biol. Chem.* **66**, 145.  
 Mauthner and Suida (1894). *Monatsh. Chem.* **15**, 362.  
 Pohl (1926). *Nachr. Ges. Wiss. Göttingen*, 144.  
 Reindel, Walter and Rauch (1927). *Liebigs Annalen*, **452**, 34.  
 Reinitzer (1888). *Monatsh. Chem.* **9**, 421.  
 Rosenheim and Webster (1925). *Lancet*, i, 1025.  
 ——— (1926, 1). *J. Soc. Chem. Ind.* **45**, 932.  
 ——— (1926, 2). *Biochem. J.* **20**, 537.  
 ——— (1927, 1). *Lancet*, i, 306.  
 ——— (1927, 2). *Biochem. J.* **21**, 127.  
 Rosenthal (1922). *Monatsh. Chem.* **43**, 237.  
 Schlutz and Morse (1925). *Amer. J. Dis. Child.* **30**, 199.  
 Schulze and Winterstein (1906). *Z. physiol. Chem.* **48**, 546.  
 Smedley-MacLean and Thomas (1920). *Biochem. J.* **14**, 483.  
 Steenbock and Black (1925). *J. Biol. Chem.* **64**, 263.  
 Tanret (1890). *Ann. Chim. Phys.* (6), **20**, 289.  
 ——— (1908). *Ann. Chim. Phys.* (8), **15**, 313.  
 Windaus and Grosskopf (1922). *Z. physiol. Chem.* **124**, 8.  
 Windaus and Hauth (1906). *Ber. deutsch. chem. Ges.* **39**, 4378.  
 ——— (1907). *Ber. deutsch. chem. Ges.* **40**, 3682.  
 Windaus and Hess (1927). *Nachr. Ges. Wiss. Göttingen*, 175.

# LVII. THE INFLUENCE OF AMINO-ACIDS ON HYDROLYSIS BY PANCREATIC LIPASE.

By EDWARD ROMER DAWSON.

*From the Department of Physiology, the University of Leeds.*

*(Received March 1st, 1927.)*

THE effect of amino-acids on pancreatic lipase has been studied as a preliminary to a general investigation of the influence of proteins on hydrolysis by this enzyme, following results previously reported that certain proteins may influence the mode of action of lipase. It was found [Platt and Dawson, 1925] that the enzyme exerts preferentially an "esterase" or "lipase" action when associated with albumin and globulin respectively.

Amino-acids have been shown to increase the action of certain enzymes; Jacoby and Umeda [1914] found that glycine, alanine, leucine, tyrosine and glutamic acid accelerate hydrolysis by soya-bean urease. This promoter effect is apparently specific for  $\alpha$ -amino-acids. Kato [1923], using glycine as a typical amino-acid, investigated the phenomenon in more detail and eliminated any possible effect due to the buffer value of the added amino-acid by conducting the hydrolysis in the presence of a phosphate buffer mixture. He suggests that a definite chemical combination between glycine and urease is indicated.

Sherman and his co-workers have made exhaustive investigations on the influence of amino-acids on various amylase preparations. All  $\alpha$ -amino-acids tested were found to accelerate the hydrolysis of starch by amylase, while other compounds containing an amino-group, carboxyl group or both had no influence or even an inhibitory effect on the enzyme. Sherman and Walker [1923] advance the hypothesis that the apparent accelerating power of amino-acids is due to their retarding the destruction of the enzyme, to which they ascribe a protein structure. In support of this they have shown that the amino-acids have a relatively greater action at higher temperatures, when the destruction of the enzyme by hydrolysis will be greater.

Falk and Nelson [1912] have stated that amino-acids and products of protein hydrolysis are capable of hydrolysing esters. An analysis of their results shows that the actual amounts of hydrolysis observed are small, while sufficient attention does not appear to have been given to the control of the hydrogen ion concentration. In preliminary experiments, I carried out control estimations in which ester and amino-acid were incubated without the addition of enzyme. When the change in hydrogen ion concentration, due to the addition of amino-acid to ester and buffer or ester and water mixtures, was carefully controlled, no hydrolysis of ester due to amino-acid alone could be

demonstrated. This result has been confirmed by Bosman [1926], who repeated some of Falk's experiments and proved conclusively that any hydrolysis of ester which does occur is due, not to any specific action of the amino-acids, but to the acidity developed in the medium on the addition of these compounds.

With regard to previous work on the influence of amino-acids and related substances on lipase, Magnus [1904] was unable to activate an inactive preparation of liver lipase by the addition of glycine, alanine and tyrosine, while Terroine [1920] stated that glycine and Witte's peptone have no influence on the lipolytic action of pancreatic juice. Willstätter and Memmen [1923], however, observed a small increase in the rate of hydrolysis of tributyrin by pancreatic lipase on the addition of alanine, and a large increase on the addition of the tripeptide leucylglycylglycine. The hydrogen ion concentration was controlled by adding an ammonia-ammonium chloride buffer solution to the digestion mixtures.

#### EXPERIMENTAL.

The enzyme was used either in the form of a powder prepared by treatment of pig's pancreas with acetone and ether, or as a clear glycerol extract obtained by digesting the powder with 87 % glycerol and filtering through a hardened filter-paper. Ethyl butyrate and olive oil were chosen as substrates. When using the former the activating power on an amino-acid was obtained by determining the number of cc. of  $N/10$  NaOH required to neutralise the acid formed from 0.5 cc. of ester in the presence and absence of amino-acid. The amino-acids were added from freshly prepared solutions adjusted to the correct  $p_H$ , the final concentration of amino-acid in the digestion mixture being 0.01  $M$ .

The amount of hydrolysis of olive oil was estimated by the technique of Willstätter and Waldschmidt-Leitz [1923]. The titration with alcoholic potash was carried out in an alcohol-ether mixture using thymolphthalein as indicator. Amino-acids were added to the control estimations immediately before titration, in order to correct for the titration values of these substances in alcoholic solution.

#### *Hydrolysis of ethyl butyrate.*

The first experiments with the ethyl butyrate were carried out in the presence of a phosphate buffer mixture  $p_H$  7.0. It was found that the increase in hydrolysis due to amino-acids under these conditions was small, owing to the very marked activating power of phosphates on lipase. The estimations on ethyl butyrate were, therefore, made without the addition of buffer solutions, the reaction mixtures being adjusted to  $p_H$  7.6 initially.

*Exp. 1.* Mixtures were made up containing

10 cc. water

2 cc. amino-acid solution

2 cc. suspension containing 10 mg. of enzyme

0.5 cc. ethyl butyrate.



These were incubated with continuous shaking for three hours at 37°. The results are the mean of duplicate experiments corrected for the suitable controls.

Amino-acid added (0.01 M)	Mean titration value cc.
—	2.25
Glycine	2.60
Alanine	2.60
Leucine	2.65
Aspartic acid	3.25
Glutamic acid	3.90
Histidine	5.35

*Exp. 2.* Conditions as in *Exp. 1* except that 0.5 cc. of glycerol extract 1 was used as source of enzyme.

Amino-acid added	Mean titration value cc.
—	0.88
Glycine	1.37
Alanine	1.33
Leucine	1.35
Aspartic acid	1.90
Glutamic acid	2.35
Histidine	4.10
Lysine	1.88
Ornithine	2.00

The effect of the amino-acids is more marked when the glycerol extract is used. This is probably because protein activating substances have been removed by this further purification.

### *Hydrolysis of olive oil.*

*Exp. 3.* Mixtures were made up consisting of

- 2 cc. ammonia-ammonium chloride buffer  $p_H$  9.2
- 2 cc. solution containing 10 mg. calcium chloride
- 2 cc. amino-acid solution
- 2 cc. suspension containing 10 mg. enzyme
- 4 cc. water
- 2.5 g. olive oil.

These were shaken for three minutes and incubated at 37° for a further 57 minutes. The results are expressed as the percentage of the oil hydrolysed and are the mean of duplicates corrected for controls.

Amino-acid added	% hydrolysis
—	12.7
Glycine	13.8
Aspartic acid	18.7
Glutamic acid	19.3
Arginine	12.4
Lysine	19.6
Tryptophan	13.3
Phenylalanine	14.5

*Exp. 4.* Conditions as in *Exp. 3* except that 1 cc. of glycerol extract 2 was used.

Amino-acid added	% hydrolysis
—	19.8
Glycine	22.45
Alanine	22.55
Leucine	22.55
Aspartic acid	26.7
Glutamic acid	27.9
Histidine	32.5

*Exp. 5.* Conditions as above using 0.5 cc. glycerol extract 3.

Amino-acid added	% hydrolysis
—	13.7
Aspartic acid	21.0
Proline	15.9
Tyrosine	16.1

A consideration of the above data shows that all the amino-acids tested, with the exception of arginine, accelerate the hydrolysis of ethyl butyrate and of olive oil by pancreatic lipase. The simple monoamino-monocarboxylic acids have small effects which are equal in magnitude, while histidine has the greatest promoter action of any of the substances tested. The acceleration of hydrolysis is not due to a change in the hydrogen ion concentration on the addition of amino-acid, since the  $p_H$  of the solutions was carefully controlled by colorimetric and electrometric observations.

It was found, however, that amino-acids do not influence hydrolysis by lipase in acid solution. This was shown by a similar series of experiments to those reported above, in which acetate buffer mixtures at  $p_H$  5.4 for ethyl butyrate and  $p_H$  4.7 for olive oil replaced the alkaline buffer solutions. This result suggests that amino-acids accelerate hydrolysis by stabilising the enzyme in neutral or alkaline but not in acid solutions, and a certain amount of experimental evidence has been obtained which confirms this view.

In the first place it was observed that the promoter effect of the added amino-acid is greater at higher temperatures. Thus the addition of 0.01 *M* aspartic acid raises the optimum temperature for the hydrolysis of olive oil by lipase from 37° to 44°, when the estimations are carried out in the usual manner in the presence of alkaline buffer mixtures and calcium chloride.

This indicates that amino-acids retard the destruction of the enzyme by hydrolysis, since such a decomposition, which involves the splitting up of either the lipase or of associated substances upon which the stability of the enzyme depends, will normally be greater at higher temperatures. If this explanation is correct, the enzyme when heated in the absence of substrate should retain its lipolytic activity longer when the incubation is carried out in the presence of added amino-acid than when heated in aqueous or buffer suspensions alone. To test this point mixtures of enzyme and buffer solution were made up as for the estimation of hydrolysis of olive oil; aspartic acid or histidine was added to one series, and all were incubated at 37° before adding the substrate. Aspartic acid or histidine was then added to those mixtures which did not already contain amino-acid, and the amount of hydrolysis of olive oil in one hour determined. To estimate the amount of

inactivation of enzyme which had taken place, the activity of the enzyme which had not been previously heated was determined in the usual manner.

*Exp. 6.* The mixtures incubated previous to determining the amount of hydrolysis contained

2 cc. buffer solution  $p_H$  9.2 or 4.7  
 2 cc. solution containing 10 mg.  $CaCl_2$   
 2 cc. solution containing enzyme  
 2 cc. amino-acid solution or water  
 2 cc. water.

Enzyme	$p_H$	Amino-acid added*	Hours of previous incubation	% hydrolysis		
				Previously incubated		Freshly prepared
				Alone	With amino-acid present	
A. 0.5 cc. glycerol extract	4	Aspartic acid	1	13.9	18.0	19.2
B. " "	9.2	"	21	8.1	11.1	19.2
C. 0.5 cc. glycerol extract	5	Histidine	4	11.4	14.0	14.7
D. " "	9.2	"	24	0.5	3.3	14.7
E†. 1 cc. glycerol extract	4	Aspartic acid	4	5.3	18.6	31.4
F. 0.5 cc. glycerol extract	3	"	4	5.2	5.2	7.3

\* The final concentration of amino-acid was 0.01 *M*.

† No calcium chloride was added during the initial incubation.

A consideration of the above results shows that amino-acids increase the stability of lipase in alkaline but not in acid solutions. This is in agreement with the finding that amino-acids accelerate hydrolysis by lipase in alkaline but not in acid media.

It will be observed (*Exp. 6, E*) that the destruction of lipase in alkaline solution is more rapid when calcium salts are absent. This point is being investigated further, since it is of interest in connection with the claim by Willstätter, Waldschmidt-Leitz and Memmen [1923] that lipase from any source can be completely activated by the addition of calcium chloride, albumin and an ammonia-ammonium chloride buffer mixture. It has already been shown [Platt and Dawson, 1925] that the addition of small amounts of phosphates will further increase the activity of lipase in such a medium, and during the present investigation it was found that amino-acids can replace the albumin to produce a system which has a greater activating power for lipase than that of Willstätter.

#### SUMMARY.

1. Amino-acids accelerate the hydrolysis, in alkaline or neutral but not in acid solutions, of ethyl butyrate and olive oil by pancreatic lipase.
2. The acceleration is probably due to the increased stability of the enzyme in alkaline solutions in the presence of amino-acids.

I wish to acknowledge my indebtedness to Prof. J. B. Cohen, in whose laboratory this investigation was commenced.

## REFERENCES.

- Bosman (1926). *Proc. Roy. Soc. S. Africa*, **13**, 245.  
Falk and Nelson (1912). *J. Amer. Chem. Soc.* **34**, 828.  
Jacoby and Umeda (1914). *Biochem. Z.* **68**, 23.  
Kato (1923). *Biochem. Z.* **136**, 498.  
Magnus (1904). *Z. physiol. Chem.* **42**, 149.  
Platt and Dawson (1925). *Biochem. J.* **19**, 860.  
Sherman and Walker (1923). *J. Amer. Chem. Soc.* **45**, 1960.  
Terroine (1920). *Ann. Sci. Nat.* (10), **4**, 1.  
Willstätter and Memmen (1923). *Z. physiol. Chem.* **129**, 1.  
Willstätter and Waldschmidt-Leitz (1923). *Z. physiol. Chem.* **125**, 132.  
Willstätter, Waldschmidt-Leitz and Memmen (1923). *Z. physiol. Chem.* **125**, 93.

# LVIII. THE MICRO-DETERMINATION OF METALS IN SALTS.

By HERBERT ISAAC COOMBS.

*From the Biochemical Laboratory, Cambridge.*

*(Received March 1st, 1927.)*

THE practice of using salt formation as a means of purifying compounds of biological importance is one of the most commonly employed operations in biochemical studies, and it is therefore of the greatest importance to have an easy and certain method of analysing salts. The element which is generally selected for determination is the metal: and although the process of ashing or incinerating with sulphuric acid is so simple in theory the actual practical work is not only tedious but often leads to uncertain results. The cause of this must generally be ascribed to the persistent creeping of the sulphuric acid, and when, through lack or expense of material, it is necessary to do the analysis on a micro-scale, spitting or frothing also frequently adds to the difficulties.

Pregl has found it expedient to alter his original technique on several occasions [1924, 1]; and even with his present modification [1924, 2] the author and several others (private communications) have found it difficult or even impossible to obtain correct results with many salts, although others can be analysed with ease and accuracy. This uncertainty led the author to make some alterations in the method; and using the modifications described below he has been able to carry out many dozens of analyses on the most varied salts, always obtaining accurate results with certainty.

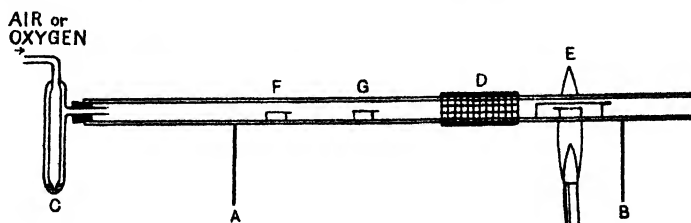


Fig. 1.



Fig. 2. (Full size.)

The chief modification consists in the use of a platinum cylinder<sup>1</sup> (Fig. 2) which surrounds the boat in which the combustion is done; any material which is generally lost by spitting or creeping is retained in the cylinder and finally weighed. The cylinder is about 40 mm. in length with a diameter of 8 mm. and is made of platinum foil 0.05 mm. in thickness. One part is flattened so that it can rest easily on a flat surface and the ends are strengthened with wire, one end also having a loop for ease of manipulation.

The cylinder and boat are boiled in dilute nitric acid (1 : 5), ignited, cooled on the block of the desiccator, placed side by side on the balance pan and weighed in the usual manner. Because of the comparatively large surface they should be allowed to remain five minutes on the pan so that they may assume a constant weight. 4 or 5 mg. of the substance to be analysed is weighed into the boat and is thoroughly moistened with two or three drops of sulphuric acid (1 : 5). It is of great advantage at this stage gently to evaporate off most of the water as this generally causes trouble in the incineration. For this purpose the boat is placed in the centre of the platinum cylinder which in turn is placed in a glass tube to protect it from dust. The whole is placed in a "regenerating" block at 120° for five minutes, a gentle current of air being drawn through the glass tube to remove the moisture.

The combustion is best carried out in a transparent silica tube (Fig. 1) about 45 cm. in length and 10 mm. internal diameter which is supported on an ordinary combustion stand *AB*. Pregl causes air to pass through the tube by using convection currents. This method has the obvious disabilities that the speed cannot be easily determined or controlled and therefore the author has found it much better to use an aspirator with a bubbler *C* to indicate the speed of the current. Moreover, when analysing salts with a high carbon content it is often difficult to burn all the carbon if only air is used and it is therefore advantageous also to have the tube connected with an aspirator containing oxygen.

After drying in the "regenerating" block the procedure of the combustion is to place the cylinder, which has the boat inside it, into the end of the silica tube, in the position shown in the figure. A fairly rapid stream of air is turned on and the flame slowly advanced from *D* to the centre of the cylinder *E*, using the wire gauze as shown to conduct the heat. This need not be done with the same great care as is necessary in the Pregl technique. After some time the gauze is removed and the full flame of the burner is allowed to play on the bare silica tube, the heat being also reflected by means of a semi-circular piece of coarse wire gauze placed over the silica tube. The air is gradually replaced by oxygen and after being heated strongly in this gas for three minutes the platinum cylinder may be removed to the desiccator, cooled and weighed as before. The course of the combustion can be watched quite well by looking through the end of the tube.

<sup>1</sup> The cylinder was made by Messrs Johnson, Matthey and Co., Ltd., Hatton Garden, London, for 28s., but the first experiments were done using a piece of foil rolled into a circular shape and this gave good results.

The author has never found any difficulty in obtaining the normal sulphates when using the above method and these give quite constant weights in air. It is obvious that if a deliquescent substance is obtained after incineration this can be weighed by placing the platinum cylinder in a suitable stoppered tube or weighing bottle after touching on the block of the desiccator. The author has also never found any reduction of the sulphates of the alkaline earths by carbon, but if there is any fear of this happening two or three drops of sulphuric acid can be distilled over the residue from small porcelain boats at *F* and *G*. In this way it is also possible to obtain excellent results from carbonates which have been obtained from ashing, etc. (cf. analyses of salt 4). Although the above experimental work has been given for incineration with sulphuric acid it is obvious that it can be used with great advantage for the generally simpler operation of ashing.

The accuracy of the method is shown by the analyses on the following pure substances (1-4). Dr H. W. Dudley was kind enough to send a few milligrams of the salts (5-8), the author only knowing the metal present. These were ordinary samples not specially purified.

No.	Salt and residue after ignition	Formula	Amount of salt taken mg.	Weight of residue mg.	Found %	Calc. %
1	Potassium tetroxalate (Potassium sulphate)	$C_4H_2O_6K, 2H_2O$	3.748 4.231	1.286 1.453	15.40 15.41	15.38
2	Potassium nitrate (Potassium sulphate)	$KNO_3$	4.747 4.434	4.086 3.821	38.62 38.67	38.68
3	Copper sulphate (Copper oxide)	$CuSO_4, 5H_2O$	4.414 4.747	1.412 1.521	25.56 25.60	25.46
4	Lithium carbonate (Lithium sulphate)	$Li_2CO_3$	3.618 4.521	5.341 6.679	18.63 18.65	18.79
5	Potassium hydrogen phthalate (Potassium sulphate)	$C_8H_5O_4K$	4.625 4.921	1.973 2.095	19.14 19.10	19.15
6	Sodium succinate (Sodium sulphate)	$C_4H_4O_4Na_2, 6H_2O$	4.715 4.296	2.479 2.252	1.702 16.97	17.03
7	Calcium oxalate (Calcium sulphate)	$CaC_2O_4, H_2O$	5.172 4.424	4.775 4.104	27.18 27.30	27.36
8	Barium benzenesulphonate (Barium sulphate)	$C_{12}H_{10}O_6S_2 Ba, H_2O$	3.778 3.487	1.884 1.733	29.35 29.25	29.25

All the analyses on the above salts were carried out as unknowns for this communication by Mr A. Colwell of this laboratory and the author's best thanks are due to him.

The author wishes to take this opportunity of thanking Prof. Pregl for his hospitality to him. He also wishes to acknowledge a grant for apparatus from the Royal Society; the work was done during the tenure of a grant from the Medical Research Council.

#### REFERENCES.

- Pregl (1924, 1). *Quantitative Organic Microanalysis*. Translated by E. Fyleman. (Churchill.  
 — (1924, 2). *Mikrochemie*, 2, 75.

# LIX. ON THE ALLEGED ACTION OF X-RAYS UPON CHOLESTEROL.

By IZRAEL HIEGER.

*From the Cancer Hospital Research Institute, London.*

*(Received March 7th, 1927.)*

THE experiments described here are part of a study of the chemical changes produced in known constituents of cells by radiations of short wave-length. The first substance chosen for investigation was cholesterol, for the reason that it is present in practically all tissues, and changes are known to be produced in it by radiations of longer wave-length (ultra-violet light). It was hoped that the investigations would throw some light upon the changes in the tissues which lead up to X-ray cancer.

## EXPERIMENTS WITH X-RAYS.

In the greater number of experiments a solution of cholesterol was placed in a glass dish 5 cm. deep and 22 cm. in diameter which was then closed, to exclude nitrogen oxides present in the X-ray room, by parchment sealed down with adhesive strapping. The dish was placed 3 or 4 cm. below the unshielded X-ray tube; the whole of the dish then lay within the cone of rays. The tube used in these experiments was a deep therapy Coolidge tube with tungsten target, activated from a coil. The current through the tube was 4 m.a. The voltages indicated in Table I were estimated by means of a sphere-gap (10 cm. diameter spheres) and checked at intervals by the spectrograph. No filter was used. The distance from the anticathode to the surface of the solution was 14 cm. The dish and tube were kept cool by an air-fan, and the solutions were exposed to X-rays for times ranging from 30 to 90 minutes, with potentials of from 100,000 to 200,000 volts (Table I).

When a thin layer (e.g. 9 mm.) of a dilute solution of cholesterol in chloroform or carbon tetrachloride is exposed to penetrating X-rays (200 k.v.) for an hour, the residue on evaporation of the solvent is found to be no longer white and crystalline as in a control sample, but to consist of an amorphous gummy mass of greenish colour and of considerably lowered melting-point (50–100° as against 146° in the original cholesterol). At first sight this result seems to reveal a striking direct effect of the rays upon the cholesterol molecule, and this conclusion has actually been drawn by one observer [Roffo, 1925; see below]. But later experiments showed that the change observed in the cholesterol is secondary to decomposition brought about by the radiation in certain chlorine-containing solvents, namely chloroform and carbon



tetrachloride; if benzene be used as solvent no such change occurs (Table I), and again no change was observed when a gum-saline emulsion of cholesterol was irradiated.

Table I.

*Action of X-rays upon solutions of cholesterol.*

Solvent	Cholesterol grams in 100 cc.	Depth of solu- tion mm.	Time of ex- posure minutes	Kilo- volts *	Melting-point		Lowering of melting- point °C.
					Irradiated ° C.	Control ° C.	
Chloroform	0.02	170	60	200	125-130	143-145	16
"	"	"	"	100	140-143	141-144	1
"	"	120	"	200	125-130	143-145	16
"	"	45	"	200	100-115	141.5-145.5	35
"	"	"	"	100	137-141	144-146	6
"	"	9.5	"	200	60-90	139-142.5	66
"	0.05	"	"	"	57-76	141-144	77
"	0.075	"	90	"	60-72	141-144	77
"	0.075	9	90	"	65-71	143-145	76
"	0.075	9	90	"	59-65	—	c. 80
"	0.05	4	30	"	100-115	140-144	34
"	0.044	"	"	175	107	—	c. 35
"	0.044	"	"	"	55	—	c. 87
Chloroform in com- pletely filled tubes	0.06	"	60	200	83-110	—	c. 45
Chloroform in current of N	0.06	5	60	200	106-113	—	c. 32
Carbon tetrachloride	0.02	170	60	200	124-131	144-145.5	17
"	0.025	5.5	30	"	70-90	140-143	62
"	"	4	30	"	60-75	—	74
"	0.05	4	60	"	60-80	139-143	71
Monochlorobenzene	0.08	7	90	200	133-138	142-146	8
Benzene	0.02	6	60	200	143-144.5	143-145	0
"	0.05	3	30	200	138-144	—	c. 2
"	0.02	6	60	100	139-142	143-145	3
Gum-saline emulsion	0.02	9	90	200	138-142	—	c. 2

*Action of radium upon solutions of cholesterol.*

Chloroform	0.1	9	2 days	50 mg. RaSO <sub>4</sub> in Pt tube	140-143	145-146.5	4
"	"	"	16 days	"	125-133	144-146	16
"	0.1	"	16 days	40 mg. RaSO <sub>4</sub> in glass tube	50-70	140-144	82
Benzene	0.1	"	2 days	50 mg. RaSO <sub>4</sub> in Pt tube	144-145	143-145	0
"	"	"	18 days	"	140-142	142-144	2

\* The wave length of the shortest X-rays present is inversely proportional to the voltage used. 200 k.v. gives "hard" rays (*i.e.* more penetrating), 100 k.v. "soft" rays (*i.e.* less penetrating).

*Details of technique.*

All the solvents used were purified by drying and fractionation. The change in the cholesterol, in a suitable solvent, is increased by reducing the depth of the solution exposed; thus in chloroform the melting-point is reduced to 125-130° when the depth is 170 mm., and to 65-70° when the depth is 9 mm., the controls recovered from non-irradiated solutions in each case melting at 143-145°. It must be remembered, however, that the dose of rays received by the solution is considerably greater in a shallow vessel, as the

scattered rays from the bottom of the vessel then penetrate the whole depth of the solution. The minimum time of exposure necessary to produce any change was not investigated. It is evident that the effect depends upon the use of high voltages, for in comparable experiments (see Table I, chloroform) a potential of 200 k.v. gave a melting-point of 100–115°, while 100 k.v. gave a melting-point of 137–141°, which is but very little lower than that of the control (144–145°).

Roffo [1925] has described this conversion of cholesterol in chloroform solution by X-rays into an amorphous greenish material, but he makes the statement that this change occurs also when benzene is used as solvent, which is in complete contradiction to the observations reported in the present paper. Now the results given in Table I above show that no such change occurred in a benzene solution; the irradiated cholesterol was recovered with no change in melting-point (143–145° in the irradiated, and 143–145° in the control, sample). Further experiments showed that the amorphous product recovered from an irradiated solution in chloroform or carbon tetrachloride could not be a simple decomposition product of cholesterol<sup>1</sup>. The product was found to possess, in addition to the very low melting-point shown by many examples in Table I, the following properties: (1) a gain in weight of not less than 5 % over that of the cholesterol taken, (2) a chlorine content of from 3.8 to 4.9 % (estimations by Bacon's method [1909]). Although, in view of the great difficulty of removing the chlorine-containing solvent, caution is needed in drawing such a conclusion, there seems to be very little doubt that a chlorinated derivative of cholesterol is formed. As soon as the presence of chlorine in the product was detected, a sample of chloroform irradiated in an atmosphere of nitrogen was tested with potassium iodide and starch and was found to contain free chlorine. Hence the conditions required for chlorination are present<sup>2</sup>. The absence of change in a benzene solution, and in a watery emulsion, seems then to be due to the absence of organically combined chlorine. In order to investigate further the importance of a halogen element, a chlorine compound of a different type, namely monochlorobenzene, was used in one experiment as solvent. The lowering of melting-point was very slight (to 133–138° in comparison with 142–146°); hence the extensive change, giving melting-points from 60 to 100°, is not brought about by all organic chlorine compounds.

The possibility was considered that the effect was due to secondary radiations from the chlorine atom. To test this, a benzene solution of cholesterol

<sup>1</sup> In attempting to purify the gummy material, great difficulty was experienced in removing the last traces of solvent. It was found best to evaporate to apparent dryness *in vacuo*, then to redissolve in ether and again evaporate *in vacuo*, and to repeat this solution in ether and evaporation five times; it was hoped that in this way the ether would carry off the last traces of the less volatile solvent. In some experiments, which gave similar results, the removal of solvent was carried out *in vacuo* at 100°.

<sup>2</sup> The boiling-point of the chloroform used was found to be lowered by about 1° after irradiation. This is in disagreement with the results of Roffo.

was poured on to a layer of solid calcium chloride, and irradiated, but no significant change in melting-point was produced. The change is not dependent upon oxidation in a solution exposed to air, for the same lowering of melting-point is observed (1) when a chloroform solution is irradiated in completely filled corked tubes and (2) when a chloroform solution is saturated with hydrogen or nitrogen and exposed to a current of the gas throughout the period of irradiation (Table I).

Further evidence of the change brought about in cholesterol in chloroform solution is afforded by (1) a change in the rotatory power from  $-46.3^\circ$  to  $-21.15^\circ$  (green Hg line), (2) a diminution in the iodine value (Wijs) from 163, an abnormally high value such as is given by cholesterol, to 102.

Before the experiments upon the irradiation of chloroform alone had shown that the change in cholesterol was due to chlorination, experiments had been begun in which the amorphous product was tested upon animals for cancer-producing power. A solution in xylene of the material was painted twice weekly on the interscapular region of two mice, and on the ear of a rabbit, for 200 days, but no tumours were produced. Roffo and Correa [1924] have described a large decrease in the cholesterol content of tumours (rat sarcoma) exposed to X-rays. Any such decomposition of cholesterol *in vivo* can hardly be due to the process, dependent upon chlorination, which occurs in certain solvents *in vitro*. Experiments upon the cholesterol content of whole animals and of separate tissues before and after irradiation are now in progress in this Institute.

#### EXPERIMENTS WITH RADIUM.

A tube of glass or platinum 0.5 mm. thick containing radium sulphate was suspended in a glass tube containing about 20 cc. of cholesterol solution, so that a thickness of about 9 mm. of solution surrounded the tube. (For details, see Table I.) The cholesterol was recovered practically unaltered from solution in benzene. With a chloroform solution, radium in a platinum tube produced practically no change ( $140-143^\circ$ ) in two days, and a distinct change ( $125-133^\circ$ ) in 16 days; when a glass tube, which permits  $\beta$ -radiation in addition to  $\gamma$ -rays to pass, was used, a product exactly similar to that produced by X-rays at high voltage was obtained, namely an amorphous material melting at  $50-70^\circ$ . Radiations from radium have therefore the same action as have X-rays upon chlorine-containing solvents. Kailan [1917] had shown that chlorine is produced by the action of radium on chloroform and carbon tetrachloride, but I was not aware of his work when the experiments described here were carried out.

The decomposition of these chlorine compounds appears to depend upon rays of wave-length less than about  $0.1 \text{ \AA}$  because (1) these would be produced by a voltage of 200 k.v. but not by 100 k.v.; as shown in Table I this voltage produces a large change in cholesterol (M.P.  $55^\circ$ ) while with a voltage of 100,000 volts there is no change in the melting-point: and (2) the screening

action of the platinum tube is most effective upon the longer  $\gamma$ -rays, and the enclosure of radium in a platinum tube greatly diminishes the effect upon cholesterol (Table I).

Some months after the experiment reported in this paper had been completed, Dognon [1926] published some qualitative observations which confirm in every way the importance of the chemical nature of the solvent, and are in direct contradiction with the findings of Roffo. Dognon employed the Liebermann test as a criterion of change in irradiated solutions of cholesterol and found (1) that the test rapidly became negative when the solvent was carbon tetrachloride, chloroform, or bromoform, and (2) that no change occurred in benzene, carbon disulphide and monochlorobenzene. The additional negative result with another chlorine-free solvent, namely carbon disulphide, is a valuable confirmation of the dependence of the change upon a halogen.

#### SUMMARY.

X-rays have been alleged to decompose cholesterol when in solution *in vitro* in various organic solvents, and it has been implied that similar changes occur *in vivo* when tumours are exposed to X-rays. The experiments described above show that X-rays act upon cholesterol only in certain chlorine-containing solvents (chloroform and carbon tetrachloride); in benzene no change is produced, and in monochlorobenzene the change is very slight. The decomposition of cholesterol in chloroform and carbon tetrachloride appears to depend upon a liberation of chlorine, the rays thus acting upon these solvents and not directly upon the cholesterol. Experiments with radium gave confirmatory results with regard to action on the solvent. In view of the dependence of the change upon chlorine-containing solvents it seems unlikely that this decomposition of cholesterol can occur in irradiated tissues.

Grateful acknowledgements are made to Dr J. E. A. Lynham of the Electrical and Radio-therapeutic Department of the Cancer Hospital for the very large number of hours which he has spent ungrudgingly in carrying out the numerous X-ray exposures. I wish to thank also Dr Leitch, the Director of this Institute, for allowing me the use of its facilities, and Dr Robert Knox, in whose Department the radiological work was carried out. This work was done during the tenure of a grant from the British Empire Cancer Campaign, to which body the author's best thanks are due, and was conducted under the supervision of Dr E. L. Kennaway, to whom he is greatly indebted.

#### REFERENCES.

- Bacon (1909). *J. Amer. Chem. Soc.* **31**, 49.  
 Dognon (1926). *Compt. Rend. Acad. Sci.* **95**, 1433.  
 Kailan (1917). *Sitzungsber. Akad. Wiss. Wien*, **II a**, **126**, 741.  
 Roffo (1925). *Compt. Rend. Acad. Sci.* **180**, 228.  
 Roffo and Correa (1924). *Z. Krebsforschung*, **22**, 79.

# LX. CONTRIBUTIONS TO THE STUDY OF BRAIN METABOLISM. IV.

## CARBOHYDRATE METABOLISM OF THE BRAIN TISSUE OF DEPANCREATISED CATS.

BY BARBARA ELIZABETH HOLMES AND ERIC GORDON HOLMES.

*From the Biochemical Laboratory, Cambridge.*

*(Received February 28th, 1927.)*

It has recently been shown by Warburg, Posener and Negelein [1924], and by Loebel [1925], that brain tissue is able to break down glucose to lactic acid with great rapidity.

We found that the lactic acid content of the excised brain (rabbit) did not rise appreciably above the "resting" level on incubation of the tissue under anaerobic conditions, although large amounts of lactic acid were formed when glucose was supplied to the tissue [Holmes and Holmes, 1925, 1]. It seemed, therefore, that the failure of the excised brain to make lactic acid must be due to a lack of glucose in the tissue.

A certain amount of reducing substance has been found in the tissue, which we have shown [Holmes and Holmes, 1926, 1] to consist mainly of creatine and creatinine, with about 30 mg. % pentose. There is no evidence for the presence of any glucose. There is a small amount of glycogen in the brain, but it does not give rise to any lactic acid, and is not lessened in amount upon incubation, whether aerobically or anaerobically, at alkaline  $p_H$  [Holmes and Holmes, 1926, 2]. E. G. H. has lately been able to show that the glycogen content of the brain tissue does not fall after depancreatisation, when the glycogen in the body tends to disappear, nor after a convulsant dose of insulin, although the brain is then suffering from a shortage of glucose. It appears, therefore, that the glycogen of the brain is in some way immobilised, and does not play the part one would expect in the carbohydrate metabolism of the brain tissue.

From consideration of these various facts we concluded that the brain contained little or no lactic acid precursor. On the other hand, it contains a by no means negligible (about 100 mg. %) amount of lactic acid after death, and the most likely origin of this lactic acid is the glucose of the blood. Though some of this glucose is probably present in the brain during life, it is all converted into lactic acid in the time (about  $3\frac{1}{2}$  minutes) elapsing between the death of the animal and the removal and rapid cooling of the brain.

If this is indeed the fact, one might expect the lactic acid content of the brain to be dependent upon the level of glucose in the blood. We have been able to show that this is apparently the case, and thus to support our view of the origin of the lactic acid in the brain.

The experiments involved are described below. The method employed for the estimation of the lactic acid is in no essential different from that of Meyerhof [1920]. Rabbits were used and the blood was obtained from an ear vein immediately before the death of the animal, which was killed by a blow on the neck. The results of the experiments are shown in Table I.

It will be seen from Table I that as the level of the blood sugar falls after the administration of insulin to the rabbit, the brain lactic acid content falls in a corresponding manner. When the hypoglycaemia is severe, the amount of lactic acid in the brain is very small. These results, which indicate that a convulsant dose of insulin produces considerable glucose deprivation in the central nervous system, are described in earlier papers [Holmes and Holmes 1925, 1, 2]. It is well to emphasise the point once more, that under these conditions of carbohydrate deprivation, the glycogen in the brain remains at a normal level. For this reason, too, the decrease in lactic acid content of the brain cannot be due to a local increase of glycogen formation in that organ, as the following figures show—a starved rabbit, just about to convulse after a dose of insulin, showed a brain lactic acid content of 36.5 mg. % and a glycogen content of 25.5 mg. %. The normal lactic acid content is 90–110 mg. %, so that, to account for the drop in lactic acid, the brain would have to contain at least 55 mg. % of extra glycogen, in addition to any which might have been present before the insulin was given.

Table I.

Exp.		Blood sugar mg. %	Brain lactic acid mg. %
1	Killed with chloroform ... ..	273	163
2	" " ether ... ..	260	159
3	Intravenous injection glucose ... ..	244	153
4	" " " " " " " " " " " "	217	146
5	" " " " " " " " " " " "	208	113
6	Anaesthetised with urethane ... ..	191	134
8	Intravenous glucose 27 mins. previously ... ..	183	123
9	Normal ... ..	160	97.4
10	" " " " " " " " " " " "	150	101
11	Insulin ... ..	124	96
12	Amytal ... ..	123	85
13	Insulin. No symptoms ... ..	107	88
14	" " No symptoms ... ..	95	76
15	" " Flaccid ... ..	65	49
16	" " Commencement of convulsion ... ..	50	45
17	Insulin convulsions well marked ... ..	—	29
18	Convulsed with insulin: recovered with glucose ...	119	56

The converse of this effect of hypoglycaemia can be seen when the blood sugar level is raised by any means. After the injection of glucose, for instance, the brain lactic acid level is high, as it is after the administration of adrenaline, or after killing with chloroform or ether, all of which processes raise the

blood glucose level. Table I gives some typical examples, showing the correspondence between the blood sugar and brain lactic acid levels.

Gesell [1925] traces a direct relationship between the level of the lactic acid in the brain, and the activity of the respiratory centre. Our experiments do not support this view, since the hyperglycaemic animals showed no hyperpnoea, while animals on the verge of insulin convulsions pant heavily.

It is interesting to notice that in deep anaesthesia the relationship between brain lactic acid and blood sugar still persists. Chloroform, ether, and urethane are known to exert a much greater inhibitory effect on the oxidative processes of brain than upon glycolysis [Loebel, 1925]. The rise in lactic acid might, therefore, be in part due to a failure in removal rather than to an increased production. The figures, however, do not suggest this, falling as they do into series with the others, nor have we been able to find any increase in cases of light amytal anaesthesia, which drug, in moderate doses at least, does not raise the blood sugar.

When the blood sugar has been abruptly raised by the injection of glucose, there is, of course, some delay before the brain lactic acid is raised correspondingly. This, doubtless, accounts for some of the irregularities in brain lactic acid level observed after the intravenous injection of glucose in normal animals, and also for the fact that in these animals the brain lactic acid level does not rise as high as it does in the case of depancreatized animals with the same blood sugar level. Exp. 18 of Table I illustrates this point, the animal having been killed a few minutes after the injection of the glucose.

Since the formation of lactic acid is known to be necessary for the process of muscular contraction, it has long been puzzling to note that there is no marked failure of this process in extreme diabetes. The problem which confronted us was a somewhat similar one. The fact that an animal will fall into convulsions when its brain is deprived of lactic acid precursor by the lowering of the blood sugar after insulin, and will recover from these convulsions when the supply of glucose is restored, indicates that the central nervous system is dependent for some purposes upon its carbohydrate metabolism. Yet, except indirectly through ketosis, there are no marked symptoms of any impairment of the functions of the central nervous system in diabetes. This was very plainly apparent in the case of our depancreatized cats, which showed no ketosis, and remained active and intelligent.

Recent work [Himwich, Loebel and Barr, 1924; Hetzel and Long, 1926] has shown that the formation of lactic acid takes place in diabetic as in normal muscles, and it is simple to show that lactic acid is present in the brain of the depancreatized animal. But whereas it is extremely difficult to determine directly the origin and fate of the lactic acid in the diabetic muscle, it should be far easier to determine the origin, at least, of the lactic acid in an organ like the brain, which has little or no precursor in its own tissues, and is dependent upon an outside supply.

We have considered the possibility that the amount of lactic acid in the

brain may depend upon the level of the lactic acid in the blood, that is, that its presence may be due in part to a passive accumulation from the blood. The figures of Himwich, Loebel and Barr [1924] do not indicate that any rise in the lactic acid level of the blood occurs as a result of diabetes, except after exercise, so that we do not need to give particular consideration to this point in connection with our depancreatized animals. We have carried out a few experiments comparing the blood lactic acid and the brain lactic acid levels, which show that, as far as the experiments on rabbits are concerned, there is no reason to suppose that the changes in the lactic acid content of the brain are due to changes in the lactic acid content of the blood.

Owing to the extreme difficulty of depancreatizing rabbits, it was decided to use cats for the following experiments. We had had no previous experience with cats, and therefore thought it necessary to carry out some determinations of the blood sugar and brain lactic acid levels in normal animals. The results (Table II) show that, in cats as in rabbits, the brain lactic acid level depends upon the blood sugar level. The normal lactic acid values are fairly constant. In two cases only (one normal and one diabetic) was there any increase in the lactic acid content of the brain after incubation; in both of these the brain had been badly injured by the blow which killed the animal. The glycogen of the brain showed no corresponding fall, and we had to assume that the breakdown of the glucose remaining in the brain had in some way been retarded. These cases supported our view that a detectable amount of the glucose entering the brain from the blood is present during life, but is usually broken down directly after death. For comparison with the other animals it seemed best to take the higher value obtained in each of these two cases, and when this was done the results agreed well with those obtained in other experiments.

Both the normal and the depancreatized animals were killed with one heavy blow on the head, death being instantaneous. The brain was removed, and was then cut in halves and one half frozen immediately, or it was quickly chopped and sampled and a portion frozen. The latter method gave the best sampling, and the slightly increased delay before freezing made no difference to the level of the "resting" values obtained. The frozen portions were weighed out and immersed in alcohol whilst still very cold, and were used for the estimations of the "resting" lactic acid content.

The estimations were carried out by the method of Meyerhof, with the following very slight modification. Since the greasiness of the tissue makes filtration through paper impossible, the alcoholic extracts were all filtered through Jena glass filters (porosity < 7 or 5-7).

For experiments dealing with the effect of aeration or of anaerobic incubation upon the lactic acid content of the tissue, the portions of the brain which had not been subjected to cooling were employed.

As we wished the depancreatized animals to be as normal as possible in every other respect, they were given injections of insulin, and sometimes of



glucose also, for the first few days after the operation<sup>1</sup>, this treatment facilitating recovery from the anaesthetic and healing of the scar. Cat 5, which was pregnant, and Cat 4 (Table II), refused to eat and were therefore killed three and five days, respectively, after the operation. Two out of the four animals operated on recovered very well (Cats 3 and 7, Table II) and these were killed eight and fourteen days respectively after the operation. At the time of death they appeared to be in good health, took a good deal of exercise, ate enormously, and were perfectly intelligent. Cat 7 excreted 5 g. of glucose per 100 cc. urine the day before its death, although it was fed entirely on lean meat with a little diluted milk. In no case could we detect any ketone bodies, by Rothera's test, in the urine of the animals, and the fact that the diabetes was uncomplicated by ketosis probably accounted in part for the surprisingly good health they displayed.

After death all the animals were very carefully examined for possible residual portions of pancreas, and any suspected fragments of tissue were taken for section. In no case, however, was any pancreatic tissue found.

As we could detect no acetone or acetoacetic acid either in the urine of the depancreatized animals or in the extracts of the brain tissue in which the lactic acid was to be estimated, we made no allowance for these substances in our estimations.

The estimations by Meyerhof's method showed the presence of a considerable amount of lactic acid, and the thiophene test for lactic acid was given by the solutions.

If this lactic acid arises from the glucose of the blood as we had found to be the case in normal animals, and if the power of the brain tissue to break down this glucose is not decreased after depancreatisation, then the greatly increased blood glucose level found in diabetic animals should lead to an increased production of lactic acid by the brain. This we did, in fact, find to be the case (see Table II).

Table II.

	Blood sugar mg. %	Brain lactic acid mg. %
Cat 5. Diabetic. Killed 3 days after depancreatisation. Pregnant; anuric for 24 hours ... ..	440	220
Cat 7. Diabetic. Killed 14 days after depancreatisation. Insulin for first 9 days. Subcutaneous injection of glucose 25 minutes before killing. Appeared healthy ... ..	317	200
Cat 4. Diabetic. Killed 5 days after depancreatisation. Would not eat. Given glucose and insulin for first 3 days. Subcutaneous injection of glucose 45 minutes before killing ... ..	311	187.5
Cat 3. Diabetic. Killed 8 days after depancreatisation. Had insulin for first 3 days. Appeared healthy ... ..	245	170
Cat 1. Killed with ether. Blood sugar certainly raised ... ..	—	127
Cat 7. Normal ... ..	123	104
Cat 8. Normal ... ..	—	90
Cat 9. Normal ... ..	110	110.5
Cat 2. Insulin. Killed in flaccid stage ... ..	—	57

<sup>1</sup> We wish to express our gratitude to Dr H. H. Dale for instructing us in the technique of this operation, and for allowing us to witness a depancreatisation which he himself performed upon a cat.

It will be seen that the brain lactic acid values correspond quite well with those obtained in normal animals when the blood sugar is artificially raised.

Thus, so far as we can judge from the data in Table II the brain tissue of a completely depancreatized cat is still capable of converting the glucose of the blood into lactic acid. A high level of glucose in the blood produces a high level of lactic acid in the brain of depancreatized as well as of normal animals. It is therefore difficult to imagine that the lactic acid which is present in the brain tissue of the depancreatized animals is derived from some unknown and abnormal source. Moreover, the excised brain tissue, taken from a completely depancreatized animal (Cat 3, 8 days after operation) was found to be capable of breaking down large amounts of glucose to lactic acid *in vitro*. The tissue was chopped and placed in Ringer's solution containing 0.25 % glucose, and potassium cyanide was added (final strength  $M/650$ ) to prevent oxidation of the lactic acid. After standing for 24 hours at room temperature, the amount of lactic acid present had increased from 171 mg. % (the "resting" value) to 365 mg. %. Tissue kept under similar conditions, but without added glucose, showed no increase in the amount of lactic acid present.

We feel safe in assuming that in all probability the origin of the lactic acid in the brain of diabetic animals is the same as that in the brain of normal animals. Of the subsequent fate of the lactic acid we know nothing, except that it is removed by some oxidative mechanism when the excised tissue is well aerated, as one would expect from Warburg's work [Warburg, Posener and Negelein, 1924], and that the power to effect this oxidative removal is retained by diabetic tissue.

The figures (Table III) showing the disappearance of lactic acid from normal and depancreatized brain tissue, illustrate this point, and also show the activity of brain tissue in oxidising preformed lactic acid. The conditions of the experiments were as follows.

The brain was chopped and weighed. One portion of it was then used immediately for the determination of the "resting" level of the lactic acid; the other portion was placed in a stoppered bottle with about 10 cc. of Ringer's solution ( $p_H$  7.4) and shaken in a bath at  $37^\circ$ , to ensure thorough aeration for about 3 hours.

Table III. *Lactic acid values expressed in mg. per 100 g. tissue.*

	"Resting" lactic acid mg.	Lactic acid after aeration mg.	Decrease mg.
Rabbit. Normal	91	15	76
Rabbit. Normal	97	36	61
Cat 6. Normal	104	41	63
Cat 4. Depancreatized	187	92	95
Cat 7. Depancreatized	202	111	91

It can be seen from this table that the power to remove lactic acid by oxidation is in no way decreased after depancreatization.

## SUMMARY.

1. The apparent absence of preformed lactic acid precursor from the brain, and the stability of the glycogen store in that organ, are discussed, and evidence is brought forward showing that the lactic acid found in the brain is formed from the glucose supplied by the blood. The lactic acid values fall and rise with the blood sugar, both in hypo- and hyper-glycaemic conditions.

2. Hyperglycaemia due to the administration of anaesthetics is accompanied by increased brain lactic acid values.

3. The brains of completely depancreatized cats show a resting lactic acid value corresponding to the degree of hyperglycaemia existing at the time of death.

4. The brain tissue of diabetic, like that of normal animals, is capable of converting glucose to lactic acid, and of removing lactic acid under aerobic conditions.

One of us [B. E. H.] is in receipt of a grant from the Medical Research Council.

## REFERENCES.

- Gesell (1925). *Amer. J. Physiol.* 75, 170.  
Hetzel and Long (1926). *Proc. Roy. Soc. Lond. B*, 99, 279.  
Holmes and Holmes (1925, 1). *Biochem. J.* 19, 492.  
—— — (1925, 2). *Biochem. J.* 19, 836.  
—— — (1926, 1). *Biochem. J.* 20, 595.  
—— — (1926, 2). *Biochem. J.* 20, 1196.  
Loebel (1925). *Biochem. Z.* 161, 219.  
Himwich, Loebel and Barr (1924). *J. Biol. Chem.* 59, 265.  
Meyerhof (1920). *Pflüger's Arch.* 182, 239.  
Warburg, Posner and Negelein (1924). *Biochem. Z.* 152, 309

# LXI. A STUDY OF THE EFFECT OF HEAT AND OXIDATION ON COD-LIVER OIL AS MEASURED BY COLOUR TESTS.

BY FRANK WOKES AND STANLEY GORDON WILLIMOTT.

(*From the Biochemical Laboratory, Cambridge, and the University of Liverpool.*)

(*Received March 4th, 1927.*)

IN a previous communication [Willimott, Moore and Wokes, 1926], the destruction of vitamin A in cod-liver oil by concentrated sulphuric acid, by phosphorus pentoxide and by ultra-violet light was followed by means of the four principal colour tests put forward for the detection of this vitamin, viz.: concentrated sulphuric acid [Drummond and Watson, 1922]; powdered phosphorus pentoxide [Fearon, 1925], arsenic trichloride [Rosenheim and Drummond, 1925] and antimony trichloride [Carr and Price, 1926].

It was found that while these reagents all gave comparable results, the last two were much more sensitive and were in agreement when applied quantitatively, a Lovibond tintometer being used to measure the intensity of blue colour, which is now regarded by many as an index of the vitamin content [Rosenheim and Drummond, 1925; Takahashi *et al.*, 1925]. It was also attempted to follow the destruction of the vitamin when the oil was aerated at 100°, but a difficulty was encountered in the darkening in colour of the oil produced by heating which interfered with the colour tests. This difficulty has since been overcome, and a detailed study has been made of the effect of aeration at different temperatures.

Most of the previous work has been carried out on butter fat as the source of vitamin A, and conflicting results have been obtained. It seems probable that these discrepancies have been increased by the fact of the wide variation in vitamin content of different samples of butter and by the fact that the fat-soluble vitamins had not then been differentiated. With the exception of Osborne and Mendel's studies [1915, 1920], the earlier work in America [Steenbock, Boutwell and Kent, 1918] and also in this country [Drummond, 1919] had suggested that the destruction of vitamin A was due to heat alone and not to oxidation. Hopkins' investigations, however, carried out in 1919 [1920, 1] gave the first clear explanation when he showed conclusively that aeration as well as heat was an essential factor in the destruction of vitamin A in butter fat [1920, 2].

Hopkins [1920, 2] found that after one hour's aeration at 120° the butter still maintained a steady though subnormal growth throughout the experimental period (11 weeks), and that even after 2 hours' aeration the vitamin

was not completely destroyed. Drummond and Coward [1920] found 3 hours' aeration at 96° sufficient for complete destruction and if this process is a chemical one, a rise of 24° might be expected to reduce the time below 2 hours. Moreover, Rosenheim and Drummond [1925] found that one hour's aeration of cod-liver oil at 100° completely destroyed its vitamin A. In view of these conflicting results we decided to investigate the effect of aeration at different temperatures above and below 100°, using colour tests to measure the content of vitamin A. Although the specificity of the latter is not yet conclusively established, our experience leads us to think that, by the adoption of certain precautions, it is possible to make them reliable with a much smaller experimental error than is the case with animal experiments. We have also found that, in any given sample of oil, partial destruction of the vitamin either by irradiation [Willimott, Moore and Wokes, 1926; Willimott and Wokes, 1927] or by aeration (unpublished results) appears to render the remainder of vitamin A present in the oil unstable, so that it continues decomposing, and nearly half may be lost during the time required to carry out an animal experiment. For these reasons we have relied mainly upon colour tests.

#### EXPERIMENTAL.

A sample of Norwegian cod-liver oil, known by feeding experiments to be potent in vitamin A, was used in this investigation. About 50 cc. were placed in a boiling-tube and heated in a water-bath until the temperature of the oil became steady at 97–98°. A brisk current of air was then drawn through at the rate of 4 to 6 bubbles per second. At the end of every 5 minutes after aeration started, a few cc. of the oil were withdrawn by means of a pipette, and transferred to tightly-stoppered bottles of non-actinic glass. During withdrawal of the samples the air current was discontinued for 15 to 20 seconds. These samples of oil which had been aerated at 98° for known periods of time were at once examined for their content of vitamin A by the four colour tests. The results, which are given in Table I, show that under the conditions of our experiment, the vitamin A in cod-liver oil is destroyed by about 75 minutes' aeration at 98°. It will be noticed that, as in our previous work on the destruction of vitamin A by irradiation, concentrated sulphuric acid and phosphorus pentoxide were found less sensitive reagents than arsenic trichloride and antimony trichloride. With these last two reagents, the initial blue colour fades gradually to green, the time taken decreasing as more of the vitamin is destroyed. The end-point was taken as the time when the change from blue to green was practically instantaneous, and was probably correct to within 5 minutes.

We next attempted to measure the vitamin content of each of these samples of aerated cod-liver oil by means of the arsenic trichloride and antimony trichloride reagents, using a Lovibond tintometer. Since publishing our previous communication [Willimott, Moore and Wokes, 1926], we have

made a thorough study of the antimony trichloride test for vitamin A, and reached further conclusions of some importance, which may be summarised as follows [Wokes and Willimott, 1927].

Table I. *Colour tests on cod-liver oil which has been aerated at 98° for different periods.*

Time aerated (minutes)	Conc. $\text{H}_2\text{SO}_4$	$\text{P}_2\text{O}_5$	$\text{AsCl}_3$	$\text{SbCl}_3$	Starch iodide test
0	++	++	++	++	-
5	++	++	++	++	+
10	+	+	++	++	+
15	+	+	++	++	+
20	+	+	++	++	+
25	+	+	++	++	++
30	±	±	+	+	++
35	-	-	+	+	++
40	-	-	+	+	++
45	-	-	+	+	++
50	-	-	+	+	+++
55	-	-	+	+	+++
60	-	-	+	+	+++
65	-	-	+	+	+++
70	-	-	±	±	++++
75	-	-	-	-	++++

NOTE. The starch iodide test was applied by putting a drop of aerated oil on a piece of filter paper previously soaked in an aqueous solution of soluble starch and potassium iodide (about 1 % of each) and then dried. If a yellow colour could be observed within 5 seconds, the test was considered to be ++++, between  $\frac{1}{2}$  and 1 minute +++, between 2 and 5 minutes ++, more than 10 minutes +.

The reaction consists of a series of colour changes—blue—yellow—red, with intermediate shades. It can be retarded by using anhydrous solvents, and working at a low temperature. In practice 15° or 16° appears to be the most suitable temperature, and it is suggested that correction be made for any wide deviation from that figure. (The temperature coefficient of the reaction is approximately 2.) The reaction is accelerated when the vitamin is rendered unstable by irradiation or aeration. The tintometer reading should be taken exactly 30 seconds after mixing, and as the colour is continually changing, the changes originating at the top of the liquid where there is exposure to air, it is necessary to mix the liquid continually with a small glass rod to ensure a uniform blend of colour. When about two-thirds of the vitamin has been destroyed, the blue colour has practically all disappeared within 30 seconds after mixing, and it is therefore impossible to obtain a tintometer reading, although the colour tests are still quite definitely positive.

Destruction of the vitamin appears to produce substances which give an *immediate* yellow colour with the reagent. We find that when working with irradiated or aerated oils it is necessary to put in more yellow units for the initial reading in order to secure an accurate colour match at the 30 second interval. If, however, a test is made on a sample of oil in which the vitamin has been completely destroyed, the colour immediately produced is red-brown. This same colour is finally reached in any test, although in a stable oil it may not be attained for over an hour.

When the concentration of cod-liver oil in the reaction mixture is plotted against blue colour produced by either arsenic trichloride or antimony trichloride, we find in the case of all the oils so far examined that the results cease to be a linear function above a concentration of 2 to 3 %. We therefore suggest that the amounts taken be arranged so that the readings of Lovibond blue units, using a half-inch cell, are not higher than 15 to 20.

The experimental findings which we have described above show the necessity of adopting various precautions when using either arsenic trichloride or antimony trichloride as reagent for vitamin A. We have now carried out some hundreds of observations on oils from Norway, Newfoundland and Iceland, and have formed the opinion that, when these precautions are observed, the experimental error on the results obtained is probably less than 10 %.

We next estimated by means of the antimony trichloride reagent the vitamin A content of each of the samples of cod-liver oil which had been aerated at 98° for different periods of time, taking in each case 0.2 cc. of a 20 % V/V solution of the oil, and mixing in a half-inch cell with 2 cc. of a solution of antimony trichloride in anhydrous chloroform, about 26 % W/V. In doing so we adopted all the precautions suggested by our previous experience. At least three readings were taken on each sample, and found to agree within 10 %. A number of readings were also obtained with arsenic trichloride, using the technique previously described [Willimott, Moore and Wokes, 1926], and were found to agree with those obtained with antimony trichloride within the limits of experimental error. The mean results are plotted in Fig. 1, in which the curve marked 98° represents the effect on vitamin A, as measured by colour tests, of aeration at 98° for different periods of time, under the conditions of our experiment. The temperature of the oil was taken every minute, and was found not to vary more than a degree either way, with an average value of 98°. The rate of aeration we had adopted was approximately the same as that of Hopkins [1920, 2]. In order to test the effect of increasing the rate of aeration, a second experiment was carried out in which the air was drawn through at a maximum speed of 10 to 12 bubbles per second (practically a continuous stream), the temperature being maintained at 98°, and results estimated as before. The results of these two experiments showed the variation to be less than the experimental error.

Further experiments were then carried out at 88°, 108°, 118°, and 125°, using a paraffin-bath in the last three cases, and adopting the same precautions. The results are plotted in continuous lines in Fig. 1. In order to estimate how much of the destruction of the vitamin was due to heat *per se*, in each experiment a blank was run by putting in the bath a test-tube of oil loosely plugged with cotton-wool, from which samples were withdrawn at 15 minute intervals. The vitamin content of each of these samples was also estimated in the same manner, and the results for each temperature are shown by the broken lines.

Turning to the effect of aeration on vitamin A, we found that the time required for complete destruction was 105 minutes at 88°, 75 minutes at 98°,

50 minutes at 108°, 35 minutes at 118°, and 30 minutes at 125°. These results agree closely with those obtained by previous workers, using both animal experiments and colour tests.

The temperature coefficient for the destruction of vitamin A by aeration, calculated from our data for each 10 degrees rise between 88° and 125°, would seem to be between 1.4 and 1.5. This is similar to the temperature coefficient of 1.5 obtained by Delf [1918] for the destruction of vitamin C by heat.

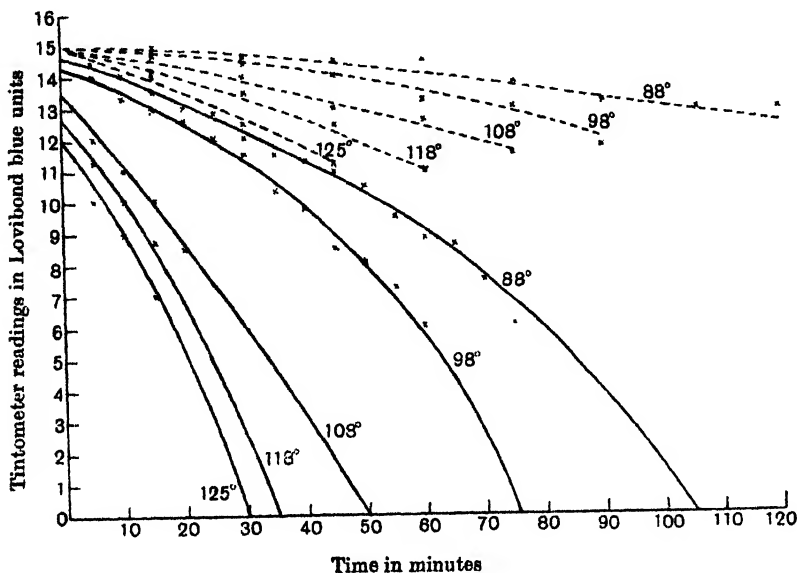


Fig. 1. Effect of heat and oxidation on vitamin A.

Similar results were obtained in the case of the oil heated without aeration at the same series of temperatures. Since there was contact with air at the small surface in the tube, the rate of destruction, although much less than that of the vitamin in the oil aerated at the same temperature, was greater than has been observed by previous workers in the case where air was strictly excluded.

In view of the opinion that the destruction of the vitamin is due to the oxygen of the air, we thought it of interest to examine the aerated oils for oxidising substances. Zilva [1920, 1922] has shown that the destructive action of ultra-violet rays is due to the ozone which they have produced in the surrounding air. Rosenheim and Webster [1926] obtained evidence of the presence of peroxides in cod-liver oil in which the vitamin A had been destroyed by exposure to air and sunlight at room temperature. In our own experiments on the destruction of vitamin A in cod-liver oil by ultra-violet light, we have been able to detect ozone in the samples of irradiated oils, after removal from the vicinity of the mercury vapour lamp, in amounts which



steadily increased as the destructive action proceeded. In the case of the destruction of the vitamin by aeration, we do not think that ozone is an active agent. Schönbein [1858] certainly suggested that ozone may be produced in the slow oxidation of various oils containing unsaturated hydrocarbons, but this suggestion was refuted by Engler [1898, 1904] who stated that the tests obtained were due, not to free ozone, but to unstable peroxides. For instance, turpentine after shaking with air will colour starch iodide paper blue, decolorise methylene blue, etc. We therefore applied, both to the aerated oils immediately after collection, and to the air which had been drawn through them, a number of tests considered characteristic of ozone, of hydrogen peroxide, and of organic and other peroxides. The results, which are summarised in Table II, would seem to indicate that the destruction of vitamin A by aeration is probably due to volatile organic peroxides. This tentative conclusion is of interest in connection with our previous suggestion [Willimott and Wokes, 1926, 2] that the distribution of vitamin A in the *Citrus* fruits may possibly be related to the presence of peroxidases.

Table II. *Tests for oxidising substances in cod-liver oil after aeration.*

Test applied to	Starch iodide	Acid KMnO <sub>4</sub>	Tetra- methyl base	Ti(SO <sub>4</sub> ) <sub>2</sub>	Guaiacum and peroxidase
Oil before aeration ...	-	-	-	-	-
Oil after aeration ...	+	+	-	-	?
Air before passage through aerated oil ...	-	-	-	-	-
Air after passage through aerated oil ...	+	+	-	-	?
Ozone ...	+	-	+	-	-
Hydrogen peroxide ...	+	+	-	+	+
Organic peroxides ...	+	+	-	-	±

#### NOTES.

When the air, after passage through the oil, was bubbled through strong chromic acid before passing into starch iodide, a negative result was obtained. This method is suggested by Mellor [1922] for differentiating between ozone (gives positive) and peroxide (gives negative).

As a source of peroxidase free from oxygenase we used an aqueous extract of the colourless residue obtained by exhausting the rind of lemons with 90 % alcohol, according to the method of Onslow [1919]. We have previously shown this to contain peroxidase only [Willimott and Wokes, 1926, 1]. The tincture of guaiacum had been boiled with charcoal to remove peroxides.

As sources of organic peroxides we used various autoxidised oils, such as turpentine and orange rind oil, heated to 98°.

#### SUMMARY.

Cod-liver oil was aerated at different temperatures between 88° and 125°, and colour tests were made at frequent intervals for vitamin A. Qualitative agreement was obtained with the four tests, concentrated sulphuric acid, phosphorus pentoxide, arsenic trichloride and antimony trichloride, and the last two gave the same end-point for complete destruction of the vitamin.

Arsenic trichloride and antimony trichloride were then applied quantitatively to estimate the vitamin. Curves were obtained representing the course of destruction of the vitamin at 88°, 98°, 108°, 118° and 125°. The temperature coefficient for 10° was found to be about 1.4 to 1.5. These results were in

agreement with observations made by other workers, using animal experiments.

The results of a preliminary series of tests applied to the oils after aeration indicated that the destruction of the vitamin may have been due to volatile organic peroxides.

We are indebted to Professor E. C. C. Baly for the loan of the tintometer.

The expenses of this investigation were in part defrayed by a grant from the Pharmaceutical Society of Great Britain.

#### REFERENCES.

- Carr and Price (1926). *Biochem. J.* **20**, 497.  
 Delf (1918). *Biochem. J.* **12**, 416.  
 Drummond (1919). *Biochem. J.* **13**, 81.  
 Drummond and Coward (1920). *Biochem. J.* **14**, 734.  
 Drummond and Watson (1922). *Analyst*, **47**, 341.  
 Engler (1898). *Ber. deutsch. chem. Ges.* **31**, 3046.  
 — (1904). Kritische Studien über die Vorgänge der Autoxidation (Braunschweig).  
 Fearon (1925). *Biochem. J.* **19**, 888.  
 Hopkins (1920, 1). *Brit. Med. J.* **ii**, 147.  
 — (1920, 2). *Biochem. J.* **14**, 725.  
 Mellor (1922). A Comprehensive Treatise on Inorganic and Theoretical Chemistry (London) **1**, 951.  
 Onslow (1919). *Biochem. J.* **13**, 1.  
 Osborne and Mendel (1915). *J. Biol. Chem.* **20**, 379.  
 — (1920). *J. Biol. Chem.* **41**, 549.  
 Rosenheim and Drummond (1925). *Biochem. J.* **19**, 753.  
 Rosenheim and Webster (1926). *Lancet*, **ii**, 806.  
 Schönbein (1858). *Ann. Chim. Phys.* (3), **52**, 221.  
 Steenbock, Boutwell and Kent (1918). *J. Biol. Chem.* **35**, 517.  
 Takahashi, Nakamiya, Kawakami and Kitasato (1925). *Inst. Phys. Chem. Research, Tokyo*, **3**, 81.  
 Willimott, Moore and Wokes (1926). *Biochem. J.* **20**, 1292.  
 Willimott and Wokes (1926, 1). *Biochem. J.* **20**, 1008.  
 — (1926, 2). *Biochem. J.* **20**, 1299.  
 — (1927). *Pharm. J.* **118**, 217.  
 Wokes and Willimott (1927). *Analyst*. (In the press.)  
 Zilva (1920). *Biochem. J.* **14**, 740.  
 — (1922). *Biochem. J.* **16**, 42.

## LXII. A NOTE ON THE AMMONIUM SULPHATE PRECIPITATION OF THE ACTIVE PRINCIPLE OF THE CULTURE FILTRATES OF *C. DIPHTHERIAE*.

By ARTHUR FREDERICK WATSON AND ELSIE LANGSTAFF.

*From the Wellcome Physiological Research Laboratories, Beckenham, Kent.*

*(Received March 1st, 1927.)*

IN previous papers [Watson and Wallace, 1924, 1, 2; Watson and Langstaff, 1926] the methods of preparation and some properties of the acid-precipitable specific principle of culture filtrates of the diphtheria bacillus have been described. The investigation has been extended to the examination of the precipitates produced by various concentrations of ammonium sulphate, the activity of the precipitates being again estimated by the *in vitro* flocculation test of Ramon [1922]. Throughout the paper the term "active principle" has been used to denote the substance (or substances) present in culture filtrates of *C. diphtheriae* which gives this specific *in vitro* flocculation with antitoxin. While it is probable that these flocculation values are by themselves rarely a reliable index of the antigenic value of the solutions, they obviously afford a valuable method of estimating the combining power of solutions obtained by chemical methods of purification of culture filtrates.

Heineman [1908] utilised the ammonium sulphate method for concentrating diphtheria toxin, and in one case obtained a solution ten times as strong as the original filtrate. Some loss of specific principle—as measured by animal tests (guinea-pig M.L.D.)—always occurred. Seibert and Long [1926], in a recent investigation of the chemical composition of the active principle of tuberculin, used glacial acetic acid and ammonium sulphate as precipitants. While the precipitation was always incomplete with the former, complete saturation with ammonium sulphate appeared to precipitate the whole of the tuberculin activity and the authors advanced experimental evidence to show that the active principle was a whole protein.

### EXPERIMENTAL.

The solutions of the crude active material were prepared, except where otherwise stated, by inoculating media made by the three-day tryptic digestion of horse muscle [Watson and Langstaff, 1927] with P.W. 8 strain of *C. diphtheriae* and growing for ten days in the incubator. The cultures were then treated with 0.5 % toluene, 0.5 % phenol or 0.75 % of neutralised formalin

(40 % formaldehyde), filtered after 24 hours and tested for Lf value<sup>1</sup>. The culture filtrates were grouped according to Lf values.

For the removal of ammonium sulphate or acid-precipitable material small volumes were filtered through paper. Where large volumes were being dealt with the precipitates were recovered quantitatively by centrifugation in a Sharples' centrifuge, the bowl of which was provided with a detachable celluloid roll, although it is of interest to note that Siebert and Long found the high specific gravity of their ammonium sulphate-tuberculin mixtures (containing glycerol) precluded this. The acid-precipitable material was dissolved in caustic soda to  $p_H$  8.0 and the sulphate-precipitable material in distilled water, in each case to the original volume or to an aliquot part. The very small amount of ammonium sulphate present in these purified solutions seemed to interfere in no way with the flocculation test with antitoxin, although it was difficult to test solutions containing more than traces of the salt owing to rapid precipitation of the serum proteins. One of the features of the testing of the more concentrated solutions was the extreme rapidity of the flocculations. Very active concentrated solutions frequently flocculate in a few minutes at 37°. On diluting these solutions the time of flocculation gradually increases, and this time is not materially altered whether the diluent is water, tryptic digest broth, muscle extract or a two per cent. solution of peptone. The organic nitrogen of the purified solutions containing small amounts of ammonium sulphate was estimated after removing the ammonia with potassium bicarbonate [see Haslam, 1905].

1. *The precipitation of the active principle at various levels of ammonium sulphate concentration.*

To equal volumes of culture filtrates which had been treated with formalin, readjusted to  $p_H$  8.0, filtered through a Berkefeld candle and incubated for a week at 36°, varying amounts of ammonium sulphate were added and the precipitates produced at the complete, two-thirds, one-half and one-third saturation levels were examined. The mixtures were allowed to stand overnight before filtration. The precipitates were washed with ammonium sulphate solution at the corresponding level and then dissolved in water. Estimations of the organic nitrogen and the specific activity of the solutions were made.

The filtrates from these initial precipitations were brought up to complete saturation by addition of solid ammonium sulphate and the second precipitates were washed, collected, dissolved in water, and tested as before.

It will be seen from Table I that the maximum recovery of the active principle is effected by complete saturation with ammonium sulphate, although similar recoveries can be made by precipitation in two successive

<sup>1</sup> As used in this paper the Lf dose may be defined as that volume of any solution containing the active principle of the culture filtrates of *C. diphtheriae* which is equal to one unit of antitoxin by the flocculation test (for fuller definition and description of the test see Glenny and Okell [1924] and Glenny and Wallace [1925]).

stages as shown. For the toxoid under consideration, however, the most pure active precipitate was obtained by two-thirds saturation—the precipitate containing 162 Lf units per mg. of nitrogen. The original toxoid contained 4 Lf units per mg. of nitrogen, so that a relative nitrogen purification of over 40 was effected.

Table I. *Precipitation of a diphtheria toxoid at various levels of saturation with ammonium sulphate.*

Original toxoid. TME 146 containing 14,000 Lf units per l. and 4 Lf units per mg. N.

Degree of saturation	Initial recovery of Lf units				Recovery of Lf units by completing saturation of filtrates from first precipitate			
	Lf units pptd. from 1 l. toxoid	Recovery %	Organic N pptd. from 1 l. (mg.)	Lf units per mg. N of ppt.	Lf units pptd. from 1 l. toxoid	Recovery %	Organic N pptd. from 1 l. (mg.)	Lf units per mg. N of ppt.
Complete	11,700	84	248	47	—	—	—	—
$\frac{3}{4}$	11,600	82	71	162	200	1	102	2
$\frac{1}{2}$	2,600	19	62	42	9,600	69	172	56
$\frac{1}{3}$	0	0	14	0	11,700	84	382	31

2. *The activity of the precipitates produced from one toxoid, (a) by varying the hydrogen ion concentration with acetic acid, (b) by saturating with ammonium sulphate.*

Experiments were next carried out to compare the efficiency of acetic acid and ammonium sulphate as precipitants. A typical experiment may be described. A volume of diphtheria toxoid was prepared and the acetic acid precipitation zone was determined, as described in a previous paper [Watson and Langstaff, 1926]. The  $p_H$  limits of this zone were found to be 3.1 to 4.35. Four litres of the toxoid were then precipitated at each of eight points within this zone and the precipitates recovered quantitatively by means of a Sharples' centrifuge. The total solids, total nitrogen content and the specific activity of the precipitate dissolved in alkali to  $p_H$  8.0 were then estimated. A similar volume of the same toxoid ( $p_H$  7.9) was completely saturated with ammonium sulphate and the activity of the precipitate dissolved in water estimated in the same way. The results are given in Fig. 1.

By varying the hydrogen ion concentration of the toxoid, maximum precipitation was produced at  $p_H$  3.9, the isoelectric point of the active "protein" (Curve A). Correlated with this, the precipitate at this point was most active (Curve B). Curve C shows the concentration of Lf units per 20 mg. nitrogen of the precipitates produced at various points in the  $p_H$  zone 3.1–4.35. The maximum activity was 4100 units per 20 mg. nitrogen, and since the original toxoid contained 37 units per 20 mg. nitrogen a relative purification of 110 was effected by precipitation at the isoelectric point. Even at this point, however, only 39 % of the total active principle was recovered.

On the other hand, by completely saturating the toxoid ( $p_H$  7.9) with ammonium sulphate, the whole of the activity was recovered in the precipitate (Curve D). As Seibert and Long [1926] have found with tuberculin, ammonium

sulphate is a far more energetic precipitant than acetic acid even at the isoelectric point. In fact a very strong resemblance (apart from heat stability) between the behaviour of the active principle of tuberculin and a true bacterial toxin such as diphtheria toxin may eventually be proved to exist.

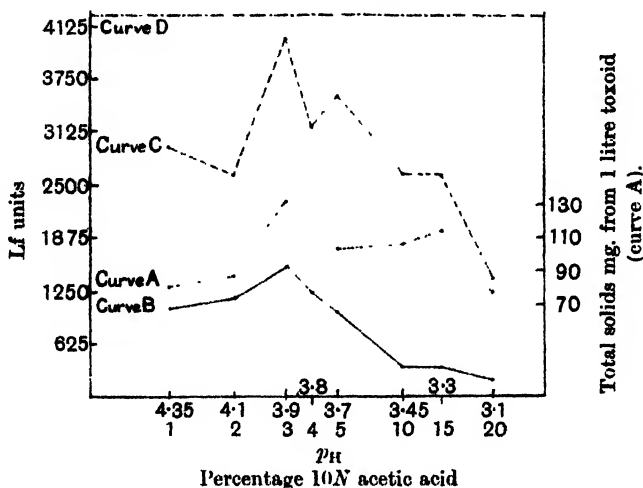


Fig. 1. The precipitability of a diphtheria toxoid by change of  $pH$  with acetic acid and by ammonium sulphate.

Curve A. Total solids precipitated from 1 l. of toxoid at various  $c_H$ .

Curve B. Specific activity (Lf units) of various precipitates.

Curve C. Lf units per 20 mg. N of the precipitates.

Curve D. Specific activity (Lf units) of the precipitate produced by complete saturation of the toxoid ( $pH$  7.9) with ammonium sulphate.

### 3. *The influence of the preservative used for culture filtrates on the precipitability of the active principle.*

The particular chemical agent used for the destruction of the bacilli at the end of growth and the preservation of the culture filtrates during subsequent manipulation may exert a definite influence on the recovery of the active principle by precipitation with ammonium sulphate. The chief agents employed for this purpose are phenol, formaldehyde and toluene, but of these, toluene is the only one that apparently has no action on the active principle. Table II shows the recoveries from a series of culture filtrates treated with different preservatives.

In all cases except two (TB 182 and TX 179—beef-heart peptone media) the media were prepared by the three-day tryptic digestion of horse muscle [Watson and Langstaff, 1927]. In the case of the filtrates treated with phenol, very little of the active principle is found in the precipitate, although examination of the amounts of nitrogen in the material precipitated in the case of unpreserved, toluene- or phenol-treated culture filtrates gave the same results. These low recoveries from phenol-treated filtrates may be due to the "salting out" of the antiseptic and the high concentration localised in the precipitate effecting severe destruction of the active principle [see Pope, 1927]. The

recoveries from unpreserved or toluene-treated filtrates are uniformly high and in some cases practically complete. On the other hand, the formalin-treated filtrates fall into two groups, one giving practically complete recovery and the other comparatively small. In every case where the yields were high the parent toxoid was well matured—i.e. the formalin had been in contact with the culture filtrates for a prolonged period and the amount of free formalin had been reduced to a minimum. Further, the  $p_H$  of the toxoid had been readjusted to 8.0. The low recoveries were produced from culture filtrates which had been in contact with formalin for a short time only. It would appear that the contributing cause to the low recoveries in these cases is a  $p_H$  effect. This effect is examined in the next section.

Table II. *The effect of the presence of preservative on the activity of the precipitate produced by complete saturation with ammonium sulphate.*

No.	Preservative	No. of Lf units per 100 cc.	Lf units recovered by complete saturation with (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Percentage yield
TME 172	Formalin	1100	1050	96
TME 93	"	425	425	100
CES 171 A	"	1150	550	48*
TME 150	"	1050	1000	95
TTE 154	Toluene	850	750	88
CES 169 A 3	"	1900	1800	95
TTE 181	"	1400	1200	86
TE 178 A	Phenol	1400	350	25
TE 177	"	1600	500	31
TB 182	"	1100	200	18
TX 179	Unpreserved	1200	1200	100
"	Toluene	1200	1200	100
"	Formalin	1200	600	50*
"	Phenol	1200	200	17

\* Formalin added a few hours before saturation with ammonium sulphate.

#### 4. *The influence of the hydrogen ion concentration of the culture filtrates on the "salting out" effects of ammonium sulphate.*

Very few data exist in the literature on the salting out of proteins (except perhaps those of serum) by ammonium sulphate. It is probable that the effects produced with any one protein are characteristic only for that protein. With the object of testing the effect of salting out the *diphtheriae* "proteins" in acid and alkaline solutions, a series of culture filtrates were adjusted to different  $p_H$  and then saturated with ammonium sulphate. The activity of the precipitates was tested in the usual way. The results are collected in Fig. 2.

It will be seen that complete recovery of the active principle is usually found if the salting out is conducted in slightly alkaline solution ( $p_H$  8.0). At points on the other side of neutrality the recovery may not be complete. In the acid precipitation zone ( $p_H$  ca. 4.0) very low recoveries are always experienced. This is probably caused by the partial acid precipitation of the

active principle in a form insoluble in water<sup>1</sup>. The whole mechanism of the salting out of proteins is an obscure one but it is of importance that the recovery by precipitation with ammonium sulphate of the active principle of diphtheria toxin in a form soluble in water is only complete in the absence of certain chemical substances (*e.g.* phenol or acid) which in some way affect the structure of the active principle.

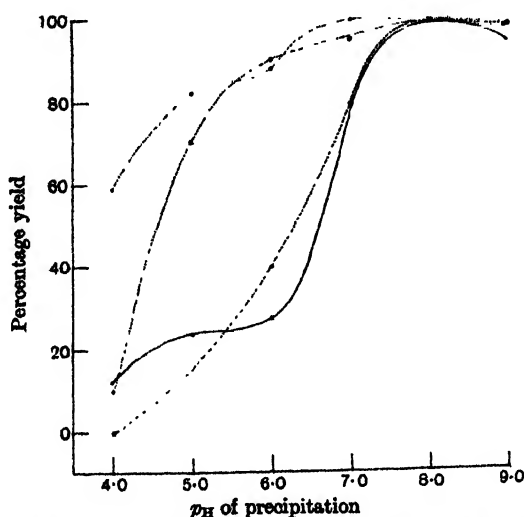


Fig. 2. The influence of  $p_H$  on the precipitation of culture filtrates by ammonium sulphate.

- Culture filtrate untreated.
- Culture filtrate treated with 0.5 % toluene.
- ..... Culture filtrate treated with 0.75 % formalin.

##### 5. The chemical "purity" of the precipitates produced by acetic acid and ammonium sulphate.

In any work involving the isolation and examination of substances of physiological interest it is obviously an advantage to utilise, as a basis for attacking the problem, material as free as possible from extraneous components. To obtain some idea of the relative purity of the precipitates during the course of the investigation, the concentrations of Lf units per mg. N of the original culture filtrates and the various precipitates have been determined. Different culture filtrates vary widely in their behaviour towards the same precipitant, but the general impression received during the course of the work was that although ammonium sulphate under certain conditions may completely precipitate the active principle from the culture filtrates, the precipitates produced in the acid precipitation zone contain relatively more Lf units per mg. N. The complete examination of a low value culture filtrate is shown in Table III. Acetic acid at the isoelectric point ( $p_H$  3.9) produced a precipitate

<sup>1</sup> Mastin and Schryver [1926] have recently described a well-defined acidic denatured product of albumin soluble in dilute alkalis but insoluble in dilute acids.



which was 110.8 times as "pure" as the original toxoid. The "purest" precipitate obtained in the ammonium sulphate series was that produced by two-thirds saturation where a relative purity of 28.8 was obtained (compare Table I, where the precipitate produced at this level also contained the highest concentration of Lf units per mg. N).

Table III. *The relative purity of the acid- and the ammonium sulphate-precipitable material.*

Toxoid TME 93. 1.85 Lf units per mg. N

Precipitate in zone $p_H$ 3.1-4.35			Ammonium sulphate precipitates		
$p_H$ of pptn.	Lf units per mg. N pptd.	Relative purification of act. principle	Degree of saturation	Lf units per mg. N pptd.	Relative purification of act. principle
4.35	150	81.1	Complete		
4.1	132	71.4		17.7	9.6
3.9	205	110.8		47.2	25.5
3.8	163	88		53.2	28.8
3.7	180	97.3		27.2	14.7
3.45	132	71.3	3	No ppt.	—
3.3	132	71.3			
3.1	74	40			

#### 6. *The chemical properties of the active substances.*

Apart from the presence of small traces of ammonium sulphate and of a larger amount of extraneous material, the chemical properties of the active substances obtained by salting out the culture filtrates are essentially similar to those of the acid-precipitable material previously described [Watson and Langstaff, 1926]. The salted out "proteins" can be freed from ammonium sulphate by dialysis, the loss of active principle depending largely on the nature of the membrane employed. The active material gives typical protein colour reactions and the specific substance is comparatively stable.

#### SUMMARY.

1. The substance which causes the specific *in vitro* flocculation with antitoxin can be salted out of culture filtrates of *C. diphtheriae* by complete saturation with ammonium sulphate. Recovery is complete in the case of filtrates untreated with any chemical agent, and adjusted to  $p_H$  8.0 before precipitation.

2. The presence of certain chemical agents (*e.g.* phenol or acid) prevents the complete recovery of the active principle by precipitation with ammonium sulphate.

3. The material salted out by ammonium sulphate is less chemically "pure" relative to the original culture filtrate than the acid precipitate produced at the isoelectric point although, in general, the chemical properties of the precipitates are the same.

## REFERENCES.

- Glenny and Okell (1924). *J. Path. Bact.* **27**, 187.  
Glenny and Wallace (1925). *J. Path. Bact.* **28**, 317.  
Haslam (1905). *J. Physiol.* **32**, 267.  
Heineman (1908). *J. Biol. Chem.* **5**, 27.  
Mastin and Schryver (1926). *Biochem. J.* **20**, 1177.  
Pope (1927). *J. Path. Bact.* **31**. (In the press.)  
Ramon (1922). *Compt. Rend. Soc. Biol.* **86**, 661, etc.  
Seibert and Long (1926). *Amer. Rev. Tuber.* **13**, 398, 408.  
Watson and Langstaff (1926). *Biochem. J.* **20**, 50.  
—— — (1927). *J. Path. Bact.* **31**. (In the press.)  
Watson and Wallace (1924, 1). *J. Path. Bact.* **27**, 271.  
—— — (1924, 2). *J. Path. Bact.* **27**, 289.

# LXIII. UNSCALED FIBRES. A NEW ASPECT OF FIBRE RESEARCH.

BY ALBERT THEODORE KING.

*From the British Research Association for the Woollen and Worsted Industries, Headingley, Leeds.*

*(Received March 9th, 1927.)*

HITHERTO both chemical and physical investigations on wool have dealt with the properties of the fibre as a whole. Conclusions drawn only from such observations as to how the structurally different parts (scale and cortex, and also medulla in the case of kempy fibres) are concerned in the total effect, can only be inferential. To resolve any property of the fibre into the contributory effects of scale and cortex requires removal of the scales in such a way as to leave the residual fibre core intact for separate investigation.

Chemical methods of separating scales and cortex almost certainly involve modifications in their chemical composition, and the examination of fibres so denuded of scales is of little value, so far as the specific effect of the cortex in the complete fibre is concerned. Mechanical separation is the only admissible method, and this can be successfully carried out in the manner described below, and as shown by the accompanying photomicrographs of fibres in various stages of removal of the scales and outer layers.

## METHOD OF UNSCALING.

Various methods were tried without success. Eventually it was found that by drawing the fibre with a small weight attached to keep it taut up and down across the edge of an ordinary microscope slide mounted horizontally, complete unscaling could be effected without breaking the fibre or damaging the bared interior. The slides require to be selected carefully. The narrow face must be smooth, and the two bounding edges require to be sharp and free from chipped places, which are easily detected when the finger nail is drawn along them. The severity of the scraping depends on the rate of rise and fall of the fibre, the departure from the vertical between the upper glass edge and the guiding eye placed above it, and the pressure on the glass edge as determined by the weight attached to the fibre. The scraping becomes more pronounced after the edge has been slightly roughened by friction with the fibre. The sound of the scraping is a good guide as to whether the operation is proceeding properly, or the edge is too much roughened for use. By slowly rotating the clip holding the upper end of the fibre while it is being raised and lowered, the weight also revolves and the whole surface becomes equally

scraped. Long coarse fibres are naturally best suited to the treatment, but with due precautions merino fibres can be unscaled with equal success. The scraped fibres have a different lustre, and no longer possess the property of "creeping" when rubbed lengthwise between the thumb and finger. This latter test affords a good indication of the progress of scale removal.

This simple apparatus meets the case where fibres require to be examined singly, for example, in swelling and elasticity investigations, and microscopic studies under various treatments, but for chemical analysis and other work requiring fibres in bulk, more rapid treatment is desirable. This can be effected by making the fibre pass a number of the glass edges suitably spaced, and by building a number of scraping units to treat several fibres simultaneously. An instrument on these lines is being developed, and will be described in a separate paper.

#### APPEARANCE OF UNSCALED FIBRES.

For the first series of photomicrographs (1, 2 and 3, Plate II), La Concordia Pure Lincoln fibres were used, these being of suitable length and uniformity, and showing a very clear scale structure in their natural condition. Euparal was used as mounting medium in preference to Canada balsam, as the former brings out the scale structure much more clearly. To give contrast the fibres were dyed (with Ponceau R.G.) before scraping.

The disappearance of scale edges and scale markings can be followed by microscopic observation. In the photographs shown, taken from a single fibre which had been subjected to thorough scraping, the middle portion of the fibre (3) is seen to have been considerably reduced in area without suffering, but rather gaining, in regularity of contour. The second photograph (2) shows a lesser degree of scraping, the unscraped end portion being shown on the extreme left (1).

The pores recently observed by Mark [1925] appear more distinctly and generally in increased numbers in the scraped portions. Probably the scales are more opaque than the cortex, and being also irregular in surface, scatter the light and obscure all but those pores immediately beneath them.

The effect of similarly treating Australian Merino fibres is shown in (4) and (5). The fibre cortex, while clearly defined and very regular, does not exhibit pores in the same striking manner as the Lincoln fibre.

The dyed fibres show scarcely any evidence of fibrillar structure in the cortex. Staining with Nile blue results in a striated appearance under the microscope suggesting narrow filaments closely packed, but not revealing any discrete cell structure. It is not impossible that these striae may be merely scratches made during the scraping, though this is regarded as improbable. The scraping is remarkably smooth under properly adjusted conditions, and fibres are rarely damaged. Where damage does occur, it is usually by lateral cuts due to horizontal vibration of the fibre along the glass edge, as shown in (6), Plate II.

By passing the fibre over a single edge and preventing any rotation, a plane surface can be produced on the fibre. By giving a half turn to the fibre clips the opposite side can be scraped, producing a thin ribbon, and interesting longitudinal sections are obtainable in this way. Number (7), Plate II, is a photomicrograph of a plane surface so produced with a La Concordia Lincoln fibre. The better definition thus obtained shows the pore structure much more clearly than is possible with a cylindrical surface.

#### APPLICATIONS.

The production of such fibres opens up a wide field of investigation into the microscopic structure, chemical composition, behaviour towards reagents, and various physical properties of the fibre cortex as distinct from the complete fibre.

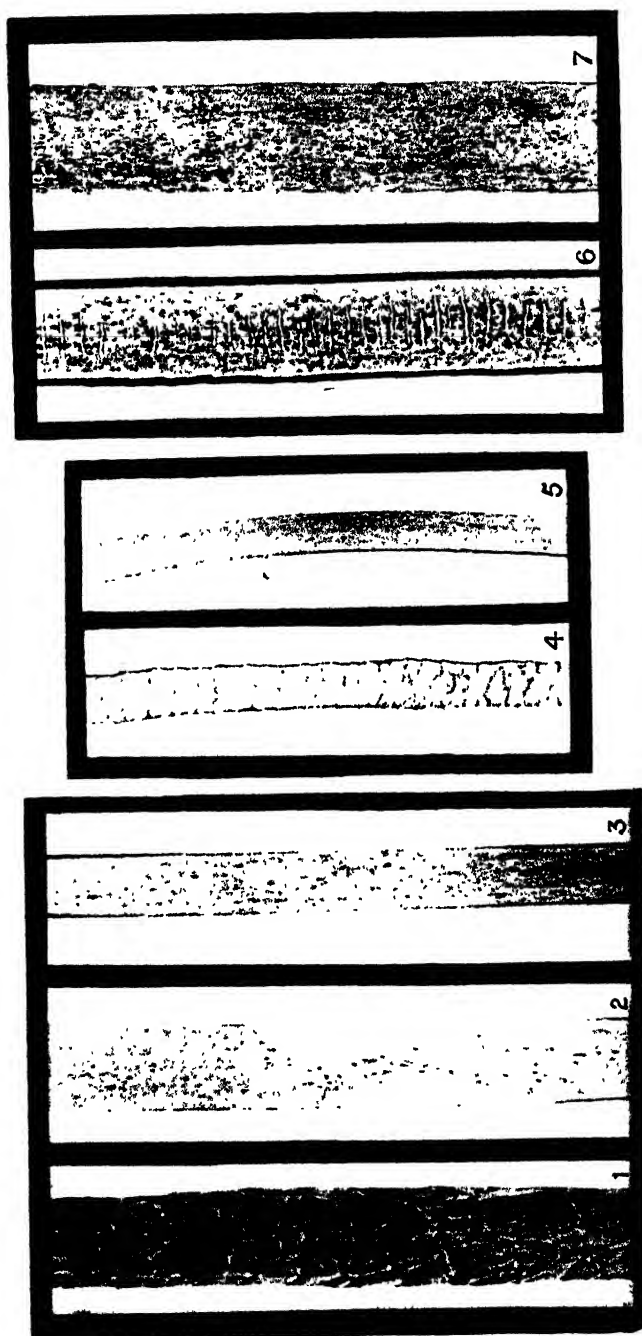
The foregoing observations are of a preliminary nature to indicate broadly the directions in which this method of attack may be of service in fibre research, on which lines investigations are now in hand.

#### SUMMARY.

A method has been devised for the mechanical removal of the scales or outer layers of wool fibres in such a way as to leave the cortex or inner core intact for physical and chemical investigations. It consists essentially in drawing the fibre across the edge of a glass plate of suitable character, or over a series of such plates suitably arranged. The effectiveness of the method is illustrated by photomicrographs of wool fibres in various stages of scale removal. The method provides a means of ascertaining the chemical and physical characters of the cortex, as distinct from those of the fibre as a whole.

#### REFERENCE.

Mark (1925). Beiträge zur Kenntniss der Wolle und ihre Bearbeitung. 32.



Magnification  $\times 300$ .



# LXIV. NOTE ON THE EFFECT OF LIGHT UPON UROPORPHYRIN.

By BERNARD THOMAS SQUIRES.

*From the Department of Biochemistry, University of Oxford.*

*(Received February 1st, 1927.)*

## INTRODUCTION.

The following paper gives some account of the phenomena occurring in relation to the action of light upon uroporphyrin, prepared from the urine of a case of haematoporphyrin congenita.

It has been known for some time that uroporphyrin fades upon exposure to daylight, and Schumm [1916] has shown that, as the fading proceeds, the colour of the solution turns from rose-red to brown, and that an additional band appears in the absorption spectrum at 4610–4620 Å.

Apart from this, little seems to be known regarding the photochemistry of uroporphyrin, although a knowledge of it must be of importance in the investigation of the part played by uroporphyrin in the sensitisation to light that is so characteristic of haematoporphyrin congenita.

## EXPERIMENTAL.

In the course of some experiments dealing with the factors involved in the process of blistering as observed in the cases of haematoporphyrin congenita, uroporphyrin solutions of different hydron concentrations were exposed to daylight, and not only did a general fading take place, but there was also some differentiation of fading between the individual solutions.

With the purpose of gaining information concerning the relation between the degree of fading and the hydron concentration of the solution, two series of experiments were performed, the one with crude uroporphyrin, the other with purified uroporphyrin.

### *(a) Daylight effects.*

*Series I.* Crude uroporphyrin was prepared by Nebelthau's method [1899] from the urine of the case of haematoporphyrin congenita described by Garrod and Mackey [1921]. This method consists essentially in the precipitation of uroporphyrin and other substances from the urine by acetic acid.

Solutions of the uroporphyrin in phosphate-buffered solutions were made up extending over the range  $p_H$  6.2–8.2. The uroporphyrin was dissolved in  $M/15$   $Na_2HPO_4$  solution in a concentration of 260 mg. per litre. To this was added, as shown in Table I, the necessary amounts of  $M/1.5$   $KH_2PO_4$  solution. In this way undue dilution of the uroporphyrin solution was avoided. (This



latter point was of importance, since colorimetric estimations had to be made, and economy had to be exercised as regards the amount of uroporphyrin used.

The  $p_H$  of each solution was confirmed by electrometric determinations.

Table I.

Solution No.	...	1	2	3	4	5
		cc.	cc.	cc.	cc.	cc.
$M/15 Na_2HPO_4 + Up.$	...	4	4	4	4	4
$M/1.5 KH_2PO_4$	...	1.6	.4	.1	.02	—
Water	...	4	1.6	1.9	1.98	2
$p_H(\pm 0.1)$	...	6.2	6.8	7.3	7.7	8.2

The solutions were in every case made up in a dark room where the stock solution of uroporphyrin in  $Na_2HPO_4$  was kept. Before exposure to light the solutions were of a bright rose-red colour.

For experiment, the solutions were exposed in sets of five in Pyrex glass tubes to daylight of varying intensity and for different times. (An ordinary photographic exposure meter was used in some cases for comparing the intensities of the different lights, but its use was afterwards discontinued, since the light rarely remained of constant intensity for more than a few minutes at a time.)

After exposure the degree of fading of the solutions was estimated colorimetrically.

Upon exposure to light a general fading always took place throughout the series, the rose colour tending to turn brownish and lighter. This fading was not due to precipitation of the uroporphyrin. Some difficulty was experienced in obtaining trustworthy colorimetric readings after exposure, as the "browning" referred to above made the colour matches with the control solutions very bad and hard to get with any great degree of accuracy. In every case, however, it was found that the solutions at  $p_H$  6.8–7.3 differed most in colour from the control tube.

*Series II.* In order to make certain upon this point, a further amount of uroporphyrin was prepared from the same source by the method of Fischer and Zerwecke [1924]. By this method uroporphyrin is obtained free from traces of coproporphyrin, and much less "browning" is observed during and after exposure, the final colour being much purer. The above experiments were then repeated exactly in detail with the purified uroporphyrin, when much more constant results were obtained, and the colour matches were far better. Table II illustrates a typical experiment.

Results obtained from an experiment using purified uroporphyrin exposed for 1 day to north light in Pyrex tubes:

Table II.

Solution No.	...	1	2	3	4	5	Control
		cc.	cc.	cc.	cc.	cc.	cc.
Absolute colorimetric reading	...	18.1	18.4	18.9	18.4	18.2	15
Reading in % increase over control	...	20.6	22.6	26.0	22.6	21.3	—
$p_H(\pm 0.1)$	...	6.2	6.8	7.3	7.7	8.2	—

*(b) Effects of ultraviolet light.*

Upon exposure of solutions, made up as above, to ultraviolet light (Cooper-Hewitt lamp) in quartz tubes, a general fading, irrespective of  $p_H$ , was obtained.

No evidence of differentiation was found with either the crude or the purified uroporphyrin.

*(c) Effects of exposure to light in absence of oxygen.*

In order to determine whether these effects might be due to oxidation, sets of solutions were sealed off in nitrogen, the technique used being similar to that described by Ellis and Newton [1925]. They were then exposed to light. After exposure gas samples from each tube were taken, and the oxygen content determined. The percentage of oxygen found was never greater than 0.1.

Under these circumstances a difference was noted between the behaviour of crude and purified uroporphyrin.

Pure uroporphyrin solutions faded with occurrence of the usual differentiation, and no difference was detected between their behaviour and that of controls set up in tubes open to the atmosphere. It would therefore appear that oxidation plays little or no part in the phenomena of fading described. Crude uroporphyrin solutions, on the other hand, fade slowly in absence of oxygen, but quickly if oxygen be present, at a much greater rate than do solutions of pure uroporphyrin. It is evident that oxidation plays some part in the fading, and it is possible that there are two reactions going on, one a purely photochemical reaction, which occurs with both purified and crude uroporphyrin, and does not involve oxidation, and the other an oxidative reaction, occurring only with the crude uroporphyrin solution.

*(d) Spectral effects.*

As the solutions fade there is little or no change in the position of the absorption-bands (of which there are four—in the solution of the purified uroporphyrin at 496–513, 533–547, 557–573, 607–618  $\mu\mu$ , and in the crude uroporphyrin at 498–525, 537–558, 565–575, 607–618  $\mu\mu$ ), at least as far as could be determined with a small direct-vision spectrometer.

The bands simply fade away as the solution fades. Within the range of  $p_H$  used the appearance of the "Kalilichtreaktion" band described by Schumm at 461  $\mu\mu$  could not be detected.

*Conclusion.*

It will be seen from the above results that the degree of fading does not increase or decrease progressively with the change in reaction of the solution. It has constantly been found that there is some definite difference in the degree of fading at  $p_H$  7.2–7.6. Though this is not great as compared with the general fading, it has been observed too constantly to be attributable to experimental error.

Whether it has any significance cannot as yet be said, but it is interesting that it should occur at a reaction which approximates to the physiological reaction. It may have some bearing upon the fact that the teeth of the patient, from whose urine the uroporphyrin was obtained, were in March 1918 (when he was two years old) of a pink colour hardly to be distinguished from that of the gums, but three years later had undergone a colour change to brownish-pink.

So far it has not been possible to obtain results in confirmation of the above by using solutions with other buffers.

#### SUMMARY.

The fading of solutions of uroporphyrin prepared from a case of haematoporphyrin congenita has been investigated within the  $p_H$  range 6.2-8.2.

1. Over this range there is a differential fading, which reaches a maximum at  $p_H$  7.3 approximately.

2. The rate of the general fading of uroporphyrin solutions is greater in the case of those containing crude uroporphyrin than in those containing purified uroporphyrin.

3. In the case of crude uroporphyrin, the fading is in part associated with the presence of oxygen; for the fading of pure uroporphyrin the presence of oxygen is not necessary.

I have to thank Prof. Sir Archibald E. Garrod and Dr L. Mackey for an introduction to the case of haematoporphyrin congenita; I am also indebted to Prof. R. A. Peters for much kindly criticism and advice.

#### REFERENCES.

- Ellis and Newton (1925). *Amer. J. Physiol.* **63**, 530.  
Fischer and Zerwecke (1924). *Z. physiol. Chem.* **137**, 176.  
Garrod and Mackey (1921). *Quart. J. Med.* **15**, 319.  
Nebelthau (1899). *Z. physiol. Chem.* **24**, 324.  
Schumm (1916). *Z. physiol. Chem.* **98**, 123.

# LXV. INFLUENCE OF GLUCOSE, ALCOHOL AND CARBON DIOXIDE AT BAROMETRIC PRESSURE ON THE $p_H$ VALUES OF PHOSPHATE AND BICARBONATE SOLUTIONS, DETERMINED BY MEANS OF HYDROQUINHYDRONE ELECTRODES.

BY EINAR BIILMANN AND HIDEO KATAGIRI.

*From the Chemical Laboratory of the University, Copenhagen.*

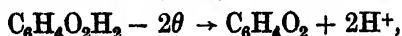
*(Received March 4th, 1927.)*

## I. INTRODUCTION.

LIQUIDS in the state of fermentation are generally saturated with  $\text{CO}_2$ . The determination of the  $p_H$  of such liquids, therefore, presents certain difficulties, though the liquids have a rather heavy buffer effect and well defined  $p_H$  values.

As a matter of fact, hydrogen electrodes cannot be used for the determination of  $p_H$  in liquids saturated with  $\text{CO}_2$  at a pressure of one atmosphere, and if colorimetric methods are used, special care must be taken to keep the liquids saturated with  $\text{CO}_2$  during the measurements.

On the other hand, it seemed possible that the quinhydrone electrode [Biilmann, 1921, 1923] would be suitable for the determination of  $p_H$  in liquids saturated with  $\text{CO}_2$ , as this electrode acts as a hydrogen electrode with a very feeble hydrogen pressure. For our purpose it will be sufficient to remember that the quinhydrone acts as a hydrogen electrode, in which hydrogen and platinum black are substituted by quinhydrone and bright platinum. The quinhydrone dissolves in water and dissociates into quinol,  $\text{C}_6\text{H}_4\text{O}_2\text{H}_2$ , and quinone,  $\text{C}_6\text{H}_4\text{O}_2$ . Quinol acts as hydrogen by the reaction

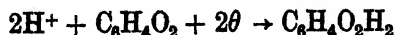


which substitutes the reaction

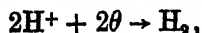


in the hydrogen electrode.

The inverse reaction



also takes place. It corresponds to the reaction



produced in the hydrogen electrode by the catalytic action of the platinum black.

In dilute solutions the potential of the quinhydrone electrode is a linear function of the  $p_H$  value, similar to that of the potential of the hydrogen electrode. At higher concentrations the influence of the solutes on the activities of quinol and quinone, formed in equimolar concentrations by the dissociation of quinhydrone, may have a certain influence on the potential, because the reversible reaction in the cell is a transformation of *dissolved* quinol into *dissolved* quinone and *vice versa*, so that the potential does not in this case depend exclusively on  $p_H$ . This influence also interferes with our experiments, which deal with solutions containing up to 10% glucose, but it is eliminated in the quino-quinhydrone electrode and the hydroquinhydrone electrode, described by Biilmann and Lund [1923]. For our purpose, we have only to mention that in the hydroquinhydrone electrode the liquid under examination is *saturated* both with quinol and with quinhydrone, and consequently, as shown in the quoted papers, the electrode reaction is here a transformation of *solid* quinol into *solid* quinhydrone. As the liquid is kept saturated with both these substances, their activities are constant, and this is why the relation between the potential and  $p_H$  is linear and independent of the concentration of other substances dissolved in the liquid.

Our experiments revealed a specific influence of glucose on the platinum plates of the electrodes, when the simple quinhydrone electrode was used. This interesting phenomenon is treated in Section VII. For the study of the influence of glucose and alcohol on the  $p_H$  values of buffer solutions and of the determination of  $p_H$  in solutions saturated with  $CO_2$ , we used the hydroquinhydrone electrode.

First of all we established, by means of phosphate buffer solutions, that the electrodes are reproduced with well-defined and stable potentials. By these experiments we arrived at a comparison electrode of the same type and almost the same  $p_H$  as the other electrodes examined, which gives our determinations the advantage that the measured cells have either no liquid junction potentials at all or only very feeble liquid junction potentials, and that the determinations do not depend on determinations of potentials between the comparison (standard) electrodes and other electrodes, but directly on the thermodynamical equation

$$p_H = p_{H_1} - \frac{\pi}{0.001983 \times T},$$

in which  $p_H$  is the  $p_H$  value of the examined liquid,  $p_{H_1}$  that of the standard electrode, and  $\pi$  indicates how much the potential of the examined electrode is higher than that of the standard electrode. Consequently, the higher the observed potential, the more acid the examined liquid, and the lower the  $p_H$  value.

## II. STANDARD HYDROQUINHYDRONE ELECTRODE.

## TEST OF METHOD.

The electrolyte of the standard electrode is a mixture of equal volumes of  $1/15\ M\ Na_2HPO_4$  and  $1/15\ M\ KH_2PO_4$ . By direct measurement its  $p_H$  value was found to be 6.79.

For the electrodes were used vessels as described by Büllmann and Lund [1923] and shown in Fig. 1. 10 cc. phosphate solution shaken with a pulverised mixture of 1 g. quinol and 0.1 g. quinhydrone were put in the vessel, and the platinum spiral was placed in the narrow part of the vessel. The electrode was left in the thermostat at  $18^\circ$  and measured against another electrode of the same kind, next day against a newly prepared electrode and so on. The two electrodes were joined by means of a  $3.5\ M\ KCl$  bridge. The results are shown in Table I.

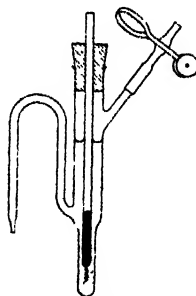


Fig. 1.

Table I.

Days	$\Delta\pi$	$\Delta p_H$
0	-0.00002	+0.003
1	+0.00040	-0.007
2	-0.00024	+0.004
4	-0.00005	+0.001

It is seen from the table that the changes in potential correspond to changes  $\Delta p_H$  which during the first 4 days are quite negligible.

Further we examined the reproducibility and steadiness of similar electrodes containing 10 g. glucose in 100 cc. Table II shows the potentials of two such electrodes measured against the phosphate standard electrode at  $18^\circ$ .

Table II.

(+)  $1/15\ M$  phosphate,  $p_H$  6.79 + glucose, 10 g. in 100 cc. |  $3.5\ M\ KCl$  |  $1/15\ M$  phosphate,  $p_H$  6.79 (-)

Volt

Minutes	I	II
0	+0.0077	+0.0079
10	+0.0074	+0.0078
30	—	+0.0078
35	+0.0074	—
65	—	+0.0078
70	+0.0075	—
1020	—	+0.0075
1080	+0.0073	—

The figures show that the maximum difference between the potentials of the two glucose-phosphate electrodes, namely between 0.0079 for electrode II at time 0 and 0.0073 for electrode I at time 1080, corresponds to less than 0.01 in the  $p_H$  value.

The reproducibility and stability of the hydroquinhydrone electrodes in a glucose-phosphate solution therefore proved to be very good.

III. INFLUENCE OF GLUCOSE ON  $p_H$  OF PHOSPHATE MIXTURE.

The figures in Table II show that glucose has an influence on the  $p_H$  value of the phosphate mixture. We therefore examined the influence of different concentrations of glucose on the phosphate mixture. The mixtures were prepared from stock solutions of primary and secondary phosphate in double concentrations (2/15  $M$ ), carbon dioxide-free water being used. Table III shows the results obtained with mixtures in which the concentration of glucose varied from 10 g. in 100 cc. to 1/32 of this value. The measurements were carried out at 18° and 25°, and the potentials recorded in the table are corrected for liquid junction potentials by means of 3.5  $M$  and 1.75  $M$  KCl. The corrections were between 0.0005 and 0.0000 volt. (It is because of this correction that the value for 10 g. glucose in Table III is higher than the corresponding value in Table II, where it is given without correction.)

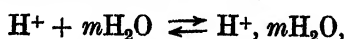
Table III.

(+) 1/15  $M$  phosphate,  $p_H$  6.79 + glucose | KCl | 1/15  $M$  phosphate,  $p_H$  6.79 (-)

g. glucose in 100 cc.	18°		25°	
	$\pi$	$\Delta p_H$	$\pi$	$\Delta p_H$
10	+0.0081	-0.140	+0.0081	-0.137
5	+0.0042	-0.073	+0.0043	-0.071
2.5	+0.0024	-0.042	+0.0023	-0.039
1.25	+0.0017	-0.029	+0.0017	-0.029
0.625	+0.0012	-0.021	+0.0012	-0.020
0.3125	+0.0008	-0.014	+0.0009	-0.015

We first observe that the electrodes *with* glucose are positive to the electrodes *without* glucose, which means that *glucose makes the mixture more acid*. The influence  $\Delta p_H$  is not negligible. The acidity of glucose itself is so feeble that glucose does not act as an acid at  $p_H$  6.79. The influence on the acidity may be explained as an influence on the activity of the water of the solutions.

Further we see that the influence on the potential is independent of the temperature. Concerning this phenomenon, it is to be remembered that as the hydration of the hydrions is expressed by the equation



we have

$$\frac{a'_{H^+}}{a'_{H_2O}} = \left( \frac{a^{\circ}_{H_2O}}{a'_{H_2O}} \right)^m = \left( \frac{P^{\circ}}{P'} \right)^m,$$

where  $a'_{H^+}$ ,  $a^{\circ}_{H^+}$ ,  $a'_{H_2O}$  and  $a^{\circ}_{H_2O}$  represent the activities of hydrions and water in two solutions,  $P^{\circ}$  and  $P'$  the water vapour pressures of these solutions.

According to the van't Hoff-Raoult law  $P^{\circ}/P'$  is independent of the temperature. The curve I in Fig. 2 shows a linear relation between  $\pi$  and g. glucose over a large range of concentrations. This is due to the fact that for the concentrations of glucose examined the values of  $\log P^{\circ}/P'$  calculated by means of the van't Hoff-Raoult law, are proportional to the concentrations:

g. glucose in 100 cc.	10	5	2.5	1.25	0.625	0.3125
$\log P^{\circ}/P' \times 10^4$	432	217	108	54	26	13

Finally we have compared the electrometric measurements with colorimetric determinations by means of bromothymol blue. We found:

g. glucose in 100 cc.	10	5	2.5	1.25	0
$p_H$ electrometric	6.65	6.72	6.75	6.76	6.79
$p_H$ colorimetric	6.71	6.75	6.78	6.79	6.79

The curve IV (Fig. 2) represents measurements made by Schreiner [1924] with saccharose in 0.005  $M$  HCl and shows the influence of saccharose on the acidity of the solution, measured by the potential of a hydroquinhydrone electrode. The curve is drawn so that the abscissae correspond to equimolar concentrations of saccharose and glucose. This curve is almost parallel with our glucose curve.

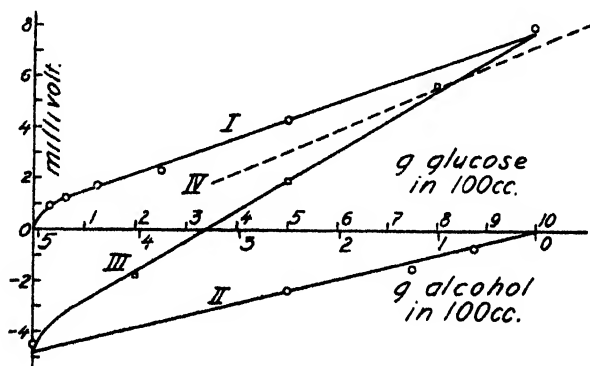


Fig. 2.

#### IV. INFLUENCE OF ALCOHOL ON THE $p_H$ OF PHOSPHATE MIXTURE.

Experiments with mixtures of phosphate solution and alcohol were made in the same way as with glucose. Regarding the amounts of alcohol formed by fermentation of glucose, alcohol was applied in concentration varying from about 5 g. in 100 cc. to 1/8 of this amount. The results are recorded in Table IV.

Table IV.

g. alcohol in 100 cc.	18°		25°	
	$\pi$	$\Delta p_H$	$\pi$	$\Delta p_H$
5.1	-0.0044	0.076	-0.0045	0.076
2.55	-0.0020	0.035	-0.0024	0.041
1.275	-0.0015	0.028	-0.0015	0.025
0.6375	-0.0006	0.010	-0.0007	0.012

The values in Table IV are corrected for liquid junction potentials. The highest extrapolation was -0.0003 volt. First we observe that the influence of alcohol is the opposite of the influence of glucose. The electrode containing alcohol is here the negative one, which means that it is less acid than the same



electrode without alcohol. So we have not to deal with an ordinary influence of a solute on the activity of the water, and the whole matter may be considered rather complicated. On the other hand, we observe here as with glucose an influence practically independent of the temperature. The influence  $\Delta p_H$  on  $p_H$  is of the same order as in the case of glucose, but has the inverse sign. In Fig. 2, curve II shows the relation between g. alcohol and  $\pi$ .

#### V. PHOSPHATE MIXTURES CONTAINING BOTH GLUCOSE AND ALCOHOL.

Finally we have examined mixtures containing both glucose and alcohol. The mixtures are composed according to the transformation of a glucose solution into alcohol by fermentation. The measurements are shown in Table V.

Table V.

1/15 <i>M</i> phosphate, $p_H$ 6.79 + glucose and alcohol   KCl   1/15 <i>M</i> phosphate, $p_H$ 6.79				
Glucose g. in 100 cc.	Alcohol g. in 100 cc.	18°	25°	25° calculated
8.0	1.02	+0.0056	+0.0057	+0.0055
5.0	2.55	+0.0020	+0.0019	+0.0020
2.0	4.08	-0.0020	-0.0018	-0.0016

The influence of glucose and alcohol is almost additive. The calculated values of  $\pi$  agree with the observed within 0.2 millivolt. For the calculation are used values obtained by graphic interpolation on curve III in Fig. 2. It represents the sum of the curve for glucose from 0 to 10 g. in 100 cc. and the curve for alcohol from 5.1 to 0 g. in 100 cc. The squares round this calculated curve show the places of the observed values of  $\pi$  from Table V.

#### VI. LIQUIDS SATURATED WITH CARBON DIOXIDE.

The following experiments deal with solutions of disodium hydrogen phosphate and of sodium bicarbonate saturated with carbon dioxide. The arrangement is shown in Fig. 3. First of all the carbon dioxide passed through a wash-bottle containing a solution of sodium bicarbonate, then a wash-bottle *a* containing some of the examined liquid, whereupon it was led into the electrode

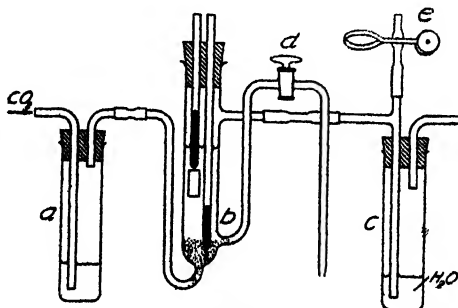


Fig. 3.

vessel *b*, which was constructed as a simple hydrogen-electrode vessel. *a* and *b* were placed in the same thermostat, so that the carbon dioxide was saturated with water vapour at such a pressure that no condensation or evaporation of water was caused by the passage of the gas through the electrode vessel. From *b* the carbon dioxide passes through the vessel *c*, which contains a little water and acts as an air-trap. If higher pressures than the actual barometric pressure were required, the carbon dioxide was led to the bottom of a high glass cylinder filled with water or salt solution.

The electrode vessel contains two electrodes: in the upper part of the liquid a platinum plate, in the lower part a platinum spiral. The latter is placed in the paste of the liquid and solid quinol and quinhydrone. For 25 cc. solution were used 3 g. of a mixture of 10 g. quinol and 1 g. quinhydrone. The liquids were saturated with  $\text{CO}_2$  before addition of the quinol-quinhydrone mixture, and then the saturation with  $\text{CO}_2$  was continued until the potentials of the platinum plate and the platinum spiral agreed within 0.0001 volt. Then the potential of the electrode was measured against the phosphate standard electrode ( $p_{\text{H}}$  6.79). If the stop-cock *d* of the electrode vessel is closed during the measurement, the conductivity will be very feeble. Therefore the carbon dioxide supply is cut off, the clip at *e* is opened for a moment, the stop-cock *d* is opened and the reading is taken. When the electrode vessel was not shaken by these manipulations, the liquid showed a constant and reproducible potential, even if it were saturated with  $\text{CO}_2$  at a pressure higher than the actual barometric pressure.

The  $\text{CO}_2$ -pressures indicated in the following tables are the pressures of dry  $\text{CO}_2$ . The potentials are corrected for liquid junction potentials in the ordinary way.

(a) *Influence of the  $\text{CO}_2$ -pressure on  $p_{\text{H}}$ .*

First we examined the influence of variations in the  $\text{CO}_2$ -pressure on the  $p_{\text{H}}$ -values of secondary phosphate solutions saturated with  $\text{CO}_2$ . The experiments were carried out with 0.1 *M*  $\text{Na}_2\text{HPO}_4$ , and the  $\text{CO}_2$ -pressure varied within the limits of the usual variations of the barometric pressure.

Table VI.

18°. 0.1 <i>M</i> $\text{Na}_2\text{HPO}_4$		
$\text{CO}_2$ , mm. Hg	$\pi$	$p_{\text{H}}$
733	+0.0200	6.444
746	+0.0204	6.437
755	+0.0214	6.419
774	+0.0224	6.402
792	+0.0230	6.392

The examined electrode is positive to the standard electrode.

In Fig. 4, the pressure of carbon dioxide is plotted against  $p_{\text{H}}$ . The relation is seen to be almost linear. None of the observed values of  $p_{\text{H}}$  differs more than 0.005 from the values given by the curve. For 0.025 *M* and 0.200 *M*  $\text{Na}_2\text{HPO}_4$  the change in  $\pi$  was of the same order.

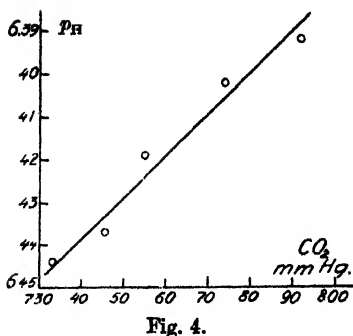


Fig. 4.



Fig. 5.

### (b) Reproducibility of the electrodes.

The reproducibility of the electrodes saturated with  $\text{CO}_2$  is shown in Table VII. Different solutions of  $\text{Na}_2\text{HPO}_4$  and of  $\text{NaHCO}_3$ , containing 5 g. glucose in 100 cc., were measured against the standard electrode with  $p_{\text{H}}$  6.79 at  $25^\circ$ .

Table VII.

$\text{Na}_2\text{HPO}_4$		mm. $\text{CO}_2$	$\pi$
0.2 M	...	733	+0.0112
		728	+0.0111
0.1 M	...	733	+0.0201
		728	+0.0192
$\text{NaHCO}_3$			
0.05 M	...	746	+0.0179
		743	+0.0178
0.025 M	...	743	+0.0335
"		738	+0.0335

The examined electrode is positive to the standard electrode.

It did not seem necessary to compare the different electrodes at exactly the same pressure, as the differences of the pressures will only correspond to one or two tenths of a millivolt. The experiments then show that electrodes saturated with  $\text{CO}_2$  are reproduced very exactly.

### (c) Influence of glucose on the $p_{\text{H}}$ of phosphate solutions saturated with $\text{CO}_2$ .

In the same way we have examined a series of solutions of  $\text{Na}_2\text{HPO}_4$ , in concentrations varying from 0.025 to 0.5 M, without glucose or with 5 g. glucose in 100 cc. The measurements were made at  $18^\circ$  and at  $25^\circ$ . Further, at  $25^\circ$  the  $p_{\text{H}}$  values of the solutions were determined with bromothymol blue (BTB) and with bromocresol purple (BCP) in order to obtain a comparison between the electrometric and colorimetric determinations of  $p_{\text{H}}$  in liquids saturated with  $\text{CO}_2$ .

The colorimetric measurement was made in the following way. 10 cc. of the solution saturated with  $\text{CO}_2$  were placed in a 20 cc. test-tube, to the bottom of which was attached a narrow tube as shown in Fig. 5. Five drops of the indicator solution were added, the test-tube was placed in the thermostat at the requisite temperature, and  $\text{CO}_2$  was bubbled through the liquid in the

test-tube (as hydrogen in a hydrogen electrode). First of all the  $\text{CO}_2$  was washed with  $\text{NaHCO}_3$  solution and then with some of the liquid under examination, as in the electrometric experiments, in order to prevent evaporation of water from the tested solution. When the saturation with  $\text{CO}_2$  was complete, the colorimetric determination was made in the ordinary way. For the comparison we used Sørensen's phosphate buffer mixtures; the exact  $p_{\text{H}}$  values of the mixtures were determined with the simple quinhydrone electrode.

The results are recorded in Tables VIII–X. In all the measurements the examined electrode was positive to the standard electrode.

Table VIII.

18°. Without glucose

Na <sub>2</sub> HPO <sub>4</sub>	mm. CO <sub>2</sub>	π	p <sub>H</sub>	735 mm. CO <sub>2</sub>	
				π	p <sub>H</sub>
0.025 <i>M</i>	...	737	+0.0431	6.04	+0.0430 6.05
0.050 <i>M</i>	...	737	+0.0306	6.26	+0.0305 6.26
0.100 <i>M</i>	...	749	+0.0204	6.44	+0.0196 6.45
0.200 <i>M</i>	...	749	+0.0122	6.58	+0.0114 6.59

The figures of the third and fourth columns give the directly measured potentials and the corresponding values of  $p_{\text{H}}$ . In order to compare the values, it is necessary to refer them to the same  $\text{CO}_2$ -pressure. This is done by means of the linear relation between the potential, or the  $p_{\text{H}}$ , and the pressure shown in Table VI and Fig. 4. It may be supposed that the small corrections are almost the same for the different concentrations of phosphate, at least within the accuracy of the measurements. The values of  $\pi$  and  $p_{\text{H}}$  referred to an average pressure of 735 mm. are recorded in the last two columns of the tables.

Table IX.

25°. Without glucose

Na <sub>2</sub> HPO <sub>4</sub>	mm. CO <sub>2</sub>	π	p <sub>H</sub>	735 mm. CO <sub>2</sub>	
				π	p <sub>H</sub>
0.025 <i>M</i>	...	728	+0.0399	6.11	+0.0403
0.050 <i>M</i>	...	728	+0.0272	6.33	+0.0276
0.100 <i>M</i>	...	722	+0.0175	6.49	+0.0182
0.200 <i>M</i>	...	722	+0.0097	6.62	+0.0104
0.500 <i>M</i>	...	735	+0.0025	6.75	+0.0025

Table X.

25°. With 5 g. glucose in 100 cc.

Na <sub>2</sub> HPO <sub>4</sub>	mm. CO <sub>2</sub>	π	p <sub>H</sub>	735 mm. CO <sub>2</sub>		
				π	p <sub>H</sub>	
0.025 <i>M</i>	...	739	+0.0410	6.10	+0.0408	6.10
0.050 <i>M</i>	...	739	+0.0292	6.30	+0.0290	6.30
0.100 <i>M</i>	...	728	+0.0192	6.47	+0.0196	6.46
0.200 <i>M</i>	...	733	+0.0112	6.60	+0.0113	6.60
0.500 <i>M</i>	...	738	+0.0040	6.72	+0.0038	6.73

The measurements show that in the case of phosphate solutions saturated with  $\text{CO}_2$ , glucose has only a very feeble influence on the potential and the  $p_{\text{H}}$ .

The differences between the potentials of solutions without and with glucose do not seem to depend on the concentration of phosphate. The variations of  $p_H$  are nearly parallel in the three series of measurements:

$\text{Na}_2\text{HPO}_4$ , $M$ ...	...	0.025	0.050	0.100	0.200	0.500
18°	$p_H$	6.05	6.26	6.45	6.59	—
25°	"	6.11	6.32	6.48	6.61	6.75
25°, with glucose	"	6.10	6.30	6.46	6.60	6.73

In the solution of secondary phosphate saturated with  $\text{CO}_2$ , we have a mixture of secondary and primary phosphate and of bicarbonate, according to the equilibrium



Experiments to be described later show that the phosphate solution saturated with  $\text{CO}_2$  does not change its  $p_H$  value so much by dilution as a pure bicarbonate solution saturated with  $\text{CO}_2$ .

We have carried out a comparison between the electrometric and the colorimetric determination of  $p_H$  in solutions saturated with  $\text{CO}_2$ . The method has already been described in the introduction to this section of our paper. For the comparison, the electrometric determinations are referred to the  $\text{CO}_2$ -pressures at which the colorimetric determinations were made. The values are recorded in Table XI.

Table XI. *Comparison of electrometric and colorimetric determinations at 25°.*

Without glucose					With 5 g. glucose in 100 cc.				
Na <sub>2</sub> HPO <sub>4</sub> <i>M</i>	mm. CO <sub>2</sub>	Electr.	<i>p</i> <sub>H</sub>		mm. CO <sub>2</sub>	Electr.	<i>p</i> <sub>H</sub>		
			Colorim.				Colorim.		
			BTB	BCP			BTB	BCP	
0.025	744	6.09	6.00	6.00	744	6.09	6.00	6.00	
0.050	729	6.33	6.22	6.22	729	6.31	6.22	6.22	
0.100	729	6.48	6.44	6.45	729	6.47	6.44	6.45	
0.200	744	6.60	6.65	6.65	744	6.59	6.65	6.70	
0.500	738	6.75	6.96	—	738	6.72	6.96	—	

The measurements show that the colorimetric method gives lower  $p_H$  values than the electrometric one up to 6.4; above this the colorimetric method gives higher values than the electrometric one.

(d) *Influence of glucose on the  $p_H$  of bicarbonate solutions saturated with  $\text{CO}_2$ .*

The experiments were made exactly as the experiments with phosphate. The bicarbonate solutions were prepared from solutions of sodium hydroxide by saturation with  $\text{CO}_2$ . The measurements are recorded in Table XII. In the last two columns of these tables are shown the  $p_H$  values referred to a pressure of 740 mm. A comparison between the  $p_H$  values of the two series of solutions shows that the influence of glucose on the acidity is very feeble, and that the bicarbonate solutions with glucose seem *less acid* than the solutions without glucose.

Table XII.

25°. Without glucose

NaHCO <sub>3</sub>		mm. CO <sub>2</sub>	$\pi$	$p_H$	$p_H$ at 740 mm.
0.025 <i>M</i>	...	738	+0.0335	6.22	6.22
0.050 <i>M</i>	...	725	+0.0190	6.47	6.46
0.100 <i>M</i>	...	756	+0.0036	6.73	6.74
0.200 <i>M</i>	...	756	-0.0109	6.97	6.98
0.500 <i>M</i>	...	755	-0.0284	7.27	7.28

With 5 g. glucose in 100 cc.

0.025 <i>M</i>	...	738	+0.0335	6.22	6.22
0.050 <i>M</i>	...	746	+0.0179	6.49	6.49
0.100 <i>M</i>	...	754	+0.0032	6.74	6.75
0.200 <i>M</i>	...	756	-0.0109	6.97	6.98
0.500 <i>M</i>	...	755	-0.0276	7.26	7.27

When we make a comparison of colorimetric and electrometric determinations of  $p_H$  (Table XIII), the last mentioned being referred to the pressures at which the colorimetric determinations are made, it shows almost the same cross point for the two methods as disodium hydrogen phosphate saturated with CO<sub>2</sub>.

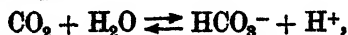
Table XIII.

Without glucose					With glucose				
NaHCO <sub>3</sub> <i>M</i>	mm. CO <sub>2</sub>	Electr.	$p_H$ Colorim.		mm. CO <sub>2</sub>	Electr.	$p_H$ Colorim.		
			BTB	BCP			BTB	BCP	
0.025	738	6.22	6.00	6.06	738	6.22	6.00	6.06	
0.050	725	6.47	6.33	6.35	725	6.50	6.33	6.35	
0.100	756	6.73	6.71	6.79	754	6.74	6.71	6.79	
0.200	756	6.97	7.00	—	756	6.97	7.00	—	
0.500	755	7.27	7.40	7.40*	755	7.26	7.40	7.40*	

\* Cresol red.

Finally we have tried to verify our determinations with bicarbonate in the following way.

The action of CO<sub>2</sub> on H<sub>2</sub>O is



whence

$$a_{\text{HCO}_3^-} \times a_{\text{H}^+} = k \times a_{\text{CO}_2} \times a_{\text{H}_2\text{O}},$$

where  $a_{\text{CO}_2}$ ,  $a_{\text{H}_2\text{O}}$ ,  $a_{\text{HCO}_3^-}$  and  $a_{\text{H}^+}$  represent the activities of CO<sub>2</sub>, H<sub>2</sub>O, HCO<sub>3</sub><sup>-</sup> and H<sup>+</sup>. If the pressure  $P_{\text{CO}_2}$  of CO<sub>2</sub> is constant, and as  $a_{\text{H}_2\text{O}}$  may be supposed to vary but very little in comparison with the other activities, we get

$$a_{\text{HCO}_3^-} \times a_{\text{H}^+} = k'.$$

For  $a_{\text{H}^+}$  we have

$$p_H = -\log a_{\text{H}^+} + 0.04.$$

As for  $a_{\text{HCO}_3^-}$ , it may be calculated from the concentrations of NaHCO<sub>3</sub> by means of the activity coefficient for HCO<sub>3</sub><sup>-</sup>. According to Warburg [1922]  $\log f_2(\text{HCO}_3^-) = -0.46 \sqrt{c}$ , where  $c$  is the stoichiometric concentration of bicarbonate.

The bicarbonate solutions are measured at different  $\text{CO}_2$ -pressures. In Table XII  $p_{\text{H}}$  values referred to 740 mm. are also to be found. This reference is made by calculation. We have at two pressures  $P_{\text{CO}_2}$  and  $P'_{\text{CO}_2}$  (when  $a_{\text{HCO}_3^-}$  is constant)

$$\frac{P_{\text{CO}_2}}{P'_{\text{CO}_2}} = \frac{a_{\text{H}^+}}{a'_{\text{H}^+}},$$

or

$$-\log a'_{\text{H}^+} = -\log a_{\text{H}^+} + \Delta,$$

where

$$\Delta = \log \frac{P_{\text{CO}_2}}{P'_{\text{CO}_2}}.$$

In Table XIV are shown the values of  $\log (a_{\text{HCO}_3^-} \times a_{\text{H}^+})$ , calculated from the experiments in Table XII.

Table XIV.

$\text{NaHCO}_3$	$a_{\text{HCO}_3^-}$	$p_{\text{H}}$	$-\log a_{\text{H}^+}$	$-\log (a_{\text{HCO}_3^-})$	$-\log (a_{\text{HCO}_3^-} \times a_{\text{H}^+})$
0.025 <i>M</i>	0.0179	6.22	6.26	1.747	8.00 <sub>7</sub>
0.050 <i>M</i>	0.0339	6.46	6.50	1.471	7.97 <sub>1</sub>
0.100 <i>M</i>	0.0612	6.74	6.78	1.214	7.99 <sub>4</sub>
0.200 <i>M</i>	0.1076	6.98	7.02	0.968	7.98 <sub>4</sub>
0.500 <i>M</i>	0.2157	7.28	7.32	0.667	7.98 <sub>7</sub>

We see that the constancy of  $-\log (a_{\text{HCO}_3^-} \times a_{\text{H}^+})$  is very good. In the calculation we presume that the change of the activity of water may be considered constant. This may not be quite right. But the influence will be very feeble. If we suppose that the influence of the bicarbonate on the activity of the water is nearly the same as of an equimolar solution of potassium chloride, and if we introduce this influence in the calculation we arrive at the following values:

$\text{NaHCO}_3$	...	...	0.025	0.050	0.100	0.200	0.500 <i>M</i>
$-\log (a_{\text{HCO}_3^-} \times a_{\text{H}^+})$	...	8.00 <sub>7</sub>	7.97 <sub>1</sub>	7.99 <sub>4</sub>	7.99 <sub>1</sub>	7.99 <sub>4</sub>	7.99 <sub>4</sub>

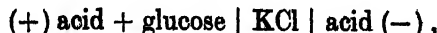
Further in a single case we have found agreement between the calculation of the influence of the  $\text{CO}_2$ -pressure on  $p_{\text{H}}$  and the influence observed experimentally. A 0.1 *M* solution of  $\text{NaHCO}_3$  was measured with the hydroquinhydrone electrode in the ordinary way.

$\text{CO}_2$ -pressure	$p_{\text{H}}$	$\Delta p_{\text{H}}$	
		Found	Calculated
742 mm.	6.669		
778 mm.	6.648	0.02 <sub>1</sub>	0.02 <sub>08</sub>

## VII. MEASUREMENTS WITH THE SIMPLE QUINHYDRONE ELECTRODE.

In the experiments described above we used the hydroquinhydrone electrode. The following were made with the simple quinhydrone electrode.

First we examined electrodes made with solutions of quinhydrone in 0.01 *N* HCl, 0.09 *N* KCl, with or without glucose. Accordingly we measured cells of the type



and we always found the electrodes with glucose to be more positive than the electrodes in the same electrolyte without glucose.

As, in the case of the simple quinhydrone electrode, there might perhaps be an influence not only on the activity of the hydron, but also on the activity of the components of the quinhydrone, we examined also the solution with the hydroquinhydrone electrode, which only shows the influence on the activity of the hydrons. The results are recorded in Table XV.

Table XV.

25°. 0.01 *N* HCl, 0.09 *N* KCl

g. glucose in 100 cc.	Quinhydrone $\pi$		Hydroquin- hydrone $\pi$
10	0.0043	0.0043	0.0041
5	0.0023	0.0022	0.0018
2.5	0.0010	0.0012	0.0012
1.25	0.0004	0.0005	—

We see that the potentials agree well and that also in the simple quinhydrone electrode we have only an influence on the activity of the hydron.

For 0.1 *N* HCl we found at 25°

10 g. glucose in 100 cc. 0.0042 volt

5                   "                   0.0021 "

values which agree completely with the figures in Table XV. The curve representing the relation between g. glucose and  $\pi$  is a straight line.

During our measurements we observed that hydroquinhydrone electrodes made with 0.01 *N* HCl, 0.09 *N* KCl are more stable in the presence of glucose than without glucose. While the hydroquinhydrone in this electrolyte was found to change 2 millivolts when kept from one day to the day after, it was quite stable in the presence of 5 % glucose. Two electrodes containing 5 g. glucose in 100 cc. of this electrolyte were kept at 18° and compared with each other and then the next two days with newly prepared electrodes of the same composition. All the examined electrodes agreed within 0.0001 volt.

When we tried to use the simple quinhydrone electrode in a phosphate mixture of  $p_H$  6.79 containing glucose, we observed that the potentials between electrodes with and without glucose were not steady but decreased gradually. In Fig. 6 are shown curves representing our measurements with solutions containing 10 g. glucose (curves I and II), 5 g. (curve III) and 1 g. (curve IV) in 100 cc. The concentration of quinhydrone was 0.0025 *M*.

The shape of the curves shows that we have not to do with a reaction between glucose and quinhydrone, the change in  $\pi$  having a higher velocity in the more dilute glucose solutions than in the more concentrated ones. By and by we convinced ourselves that the phenomenon is a specific influence of glucose on the platinum. In order to test this explanation we made the following two experiments.

1. Two platinum electrodes were kept for 3 hours at 18° in a solution of 10 g. glucose in 100 cc. and used for the measurement of phosphate  $p_H$  6.79 + 10 g. glucose in 100 cc. + quinhydrone against the same mixture without glucose. The measurements are shown in Fig. 7, curve I.



2. Two platinum electrodes were kept for 1 hour at 18° in a phosphate mixture  $p_H$  6.79 containing quinhydrone (0.0025  $M$ ) and then used for the measurement of the mixture with glucose (10 g.) against the mixture without glucose. Curve II, Fig. 7, represents the measurements.

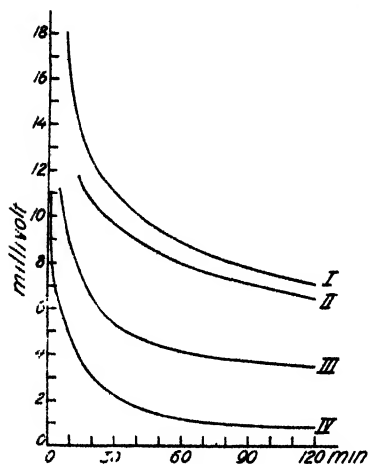


Fig. 6.

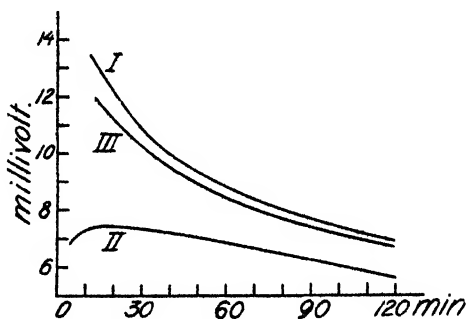


Fig. 7.

In all the electrodes the quinhydrone was 0.0025  $M$ .

The single potentials of two electrodes, in each individual case corresponding to the curves I and II, agreed well; the maximum discrepancies of the observations from the corresponding curve values were 0.8 millivolt, and most of the observations agreed within 0.2–0.4 millivolt with the curve values. But in shape the two curves are not at all like each other as will be seen from Fig. 7, in which is also drawn a curve III representing the average of the curves I and II in Fig. 6. The agreement of curves III and I in Fig. 7 is very remarkable. Evidently, in these cases, the fall of  $\pi$  must have the same origin. A similar influence of glucose was observed neither in the numerous measurements we have made with the hydroquinhydrone electrode and phosphate or bicarbonate solutions, nor in the solutions of hydrochloric acid. We point out that Warburg [1922, p. 247] observed that hydrogen electrodes in bicarbonate solutions containing saccharose did not establish the potential as rapidly as in the pure salt solutions. We shall not further discuss this rather surprising phenomenon.

#### SUMMARY.

1. The influence of glucose and of alcohol on the  $p_H$  of a phosphate mixture,  $p_H$  6.79, is examined by means of the hydroquinhydrone electrode. Glucose makes the mixture more acid, and alcohol makes it less acid. The influences of glucose and alcohol are additive.

2. Solutions of secondary sodium phosphate and sodium bicarbonate are examined with the hydroquinhydrone electrode after saturation with carbon

dioxide at barometric and higher pressures. The influence of glucose on the  $p_H$  values of the liquids saturated with carbon dioxide was examined and found to be very feeble.

3. A hydroquinhydrone electrode in a phosphate mixture,  $p_H$  6.79, was found to be very stable and suitable as a comparison electrode for measurements with hydroquinhydrone electrodes.

4. A hydroquinhydrone electrode in 0.01 *N* HCl, 0.09 *N* KCl is not very stable. It was found that it was stabilised by addition of glucose.

5. It was found that the simple quinhydrone electrode cannot be applied to phosphate solutions containing glucose. A specific action of the glucose on the platinum of the electrode was observed.

#### REFERENCES.

- Biilmann (1921). *Ann. Chem.* (9), 15, 109.  
— (1923). *Trans. Faraday Soc.* 19, 676.  
Biilmann and Lund (1923). *Ann. Chem.* (9), 16, 321.  
Schreiner (1924). *Z. anorg. Chem.* 135, 333.  
Warburg (1922). *Biochem. J.* 16, 153.



# LXVI. ON THE PHOTO-OXIDATION OF ADRENALINE.

By TOMÁŠ VACEK<sup>1</sup>.

*From the Department of Physiology and Biochemistry,  
University College, London.*

*(Received January 18th, 1927.)*

IN preliminary experiments it was observed that the presence of a very small amount of adrenaline caused blood-plasma to absorb in ultra-violet light about four times as much oxygen as the plasma alone. It was therefore decided to test the behaviour of adrenaline in ultra-violet light, employing the technique devised by Harris [1926]. Two quartz tubes, one for the adrenaline and the other the control, coupled to a Haldane volumeter were irradiated with a mercury-vapour lamp ( $\frac{1}{2}$  kw.) at a distance of 15 cm. The tubes were kept in a bath at 37°. Different preparations of adrenaline were used: adrenaline hydrochloride in solution (Parke, Davis) and pure adrenaline (Parke, Davis), dissolved either in distilled water or in 0.5 % hydrochloric acid. Amounts of 0.0005 to 0.004 g. adrenaline dissolved in 45 cc. distilled water were used.

In the first place it was determined whether oxygen absorption takes place in the dark. The volume in the adrenaline tube remained unchanged when 0.0005 to 0.004 g. adrenaline was used. Pure adrenaline, dissolved in distilled water, only showed occasionally a minute uptake of oxygen. The solution became more or less pink after a long exposure to ultra-violet radiation. The intensity of the pink colour is in direct relation to the amount of oxygen absorbed by the adrenaline.

The oxygen uptake by different amounts of adrenaline was next determined. The adrenaline hydrochloride solution in volumes from 0.5 cc. to 4 cc. was used; this solution is called A. The results are shown in Fig. 1. The higher the concentration of the adrenaline the greater the absorption of the oxygen. But the amount of the oxygen is not in direct proportion to the amount of adrenaline. The oxygen uptake by adrenaline of a higher concentration is relatively less. It is, however, probable that the absorption would be greater after a longer irradiation, and if the partial pressure of oxygen in the tubes were maintained at the same level throughout the experiments.

In the next experiments the different intensities of oxidation of different kinds of adrenaline under various conditions were tested. Here 0.001 g. of adrenaline was used and irradiated for 30 minutes. Pure adrenaline, dissolved in distilled water (C), has a very intense oxidation (Fig. 2).

<sup>1</sup> Fellow of the International Education Board.

It was further shown that hydrochloric acid is a good protective against photo-oxidation even in very low concentration (0.5 %). After an irradiation of 30 minutes adrenaline (C) took up six times more oxygen than adrenaline dissolved in 0.5 % HCl (B). And even after switching off the light the oxidation of the adrenaline C continued, though not so intensely (Fig. 2).

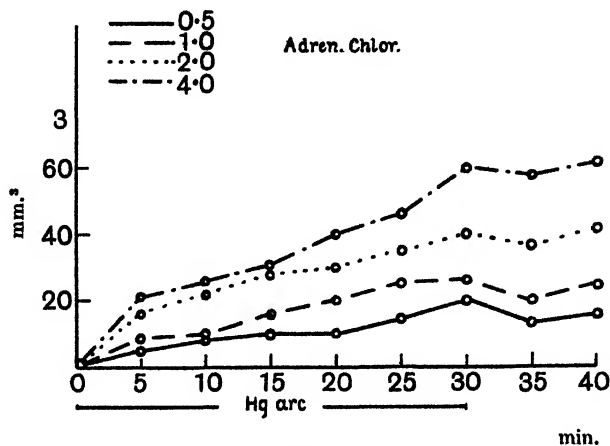


Fig. 1.

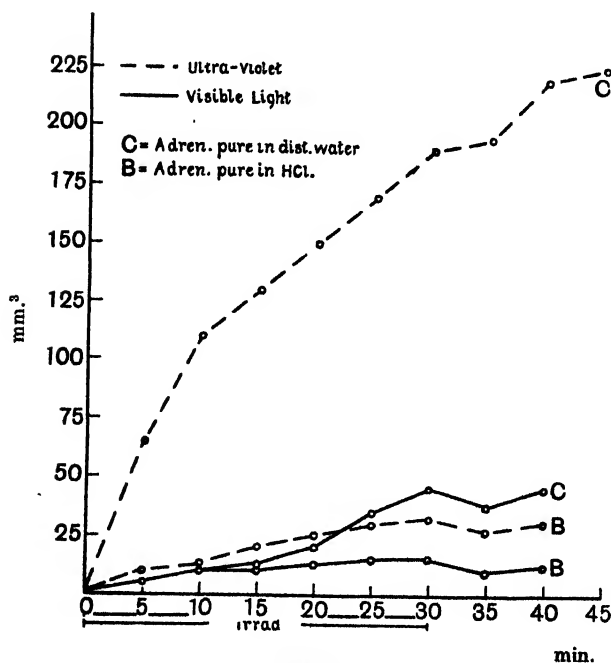


Fig. 2.

It was necessary to examine the behaviour of hydrochloric acid itself in the ultra-violet radiation. It was found that neither distilled water nor the

amount of HCl here used (1 cc. 0.5 % HCl) absorbed any appreciable amount of oxygen after a 30 min. exposure to the radiation of a mercury-vapour lamp.

It is well known that adrenaline oxidises in daylight and in these investigations it was also the object to determine the degree of oxidation in an intense light but without ultra-violet rays. In order to filter the short-wave rays a triple plate-glass filter was put between the source of the radiant energy and the quartz tubes. The two solutions (B, C) of adrenaline (0.002 g.) were examined and the results are given in Fig. 2. An oxidation took place even in the visible light, though it was much less than that caused by ultra-violet rays. The greatest uptake of oxygen in the presence of visible light only was observed in the adrenaline solution (C), whilst (B) seems to be very resistant to the influence of visible light.

#### CONCLUSIONS.

Different preparations of adrenaline show different tendencies to oxidation. Ultra-violet radiation causes an acceleration of the oxidation of adrenaline even in very large dilutions. The greatest sensitivity to light is shown by pure adrenaline in distilled water. Adrenaline hydrochloride solution shows a very low degree of oxidation.

Adrenaline exposed to visible light only behaves similarly, but the oxidation is less intense than when exposed to the short-wave rays.

The pink colour developed by adrenaline when exposed to the ultra-violet radiations is related to the uptake of oxygen. The more oxygen absorbed by the adrenaline the more pink it becomes. It would be interesting to examine the physiological action of this adrenaline, changed by the absorption of oxygen. In the paper of Azuma and Hill [1926] there appeared a short note that adrenaline, after exposure to the light, becomes inactive.

In experiments in which adrenaline from tablets (Parke, Davis, and Burroughs, Wellcome) was used, the oxygen uptake was very intense, but it is uncertain whether this may not be caused by unknown admixtures.

#### REFERENCES.

- Azuma and Hill (1926). *Proc. Roy. Soc. Lond. B*, **99**, 221.  
Harris (1926). *Biochem. J.* **20**, 271.

# LXVII. STUDIES ON THE GROWTH OF YEAST.

## IV. A NEPHELOMETRIC METHOD OF COUNTING YEAST SUSPENSIONS.

BY GEOFFREY LEWIS PESKETT.

*From the Biochemical Laboratory, Oxford.*

*Report to the Medical Research Council.*

*(Received March 17th, 1927.)*

BELOW I have recorded details of a convenient method for determining the concentration of cells in suspensions of yeast. It is based on nephelometric comparison of the yeast with standard suspensions of barium sulphate. McFarland [1907] has described a rough method for the estimation of the number of cells present in bacterial suspensions, which is essentially similar. In his earliest experiments he compared the suspensions against a dark background, but subsequently he designed an apparatus in which they were observed by direct illumination. Richards and Wells [1905], in improving the earlier method of Stas [1894], found that greater accuracy could be obtained with indirect illumination and varied the amount of light which was allowed to fall on to the tubes containing the suspensions. They determined the relative lengths of tube which were exposed to illumination when the turbidities appeared equal. In the method which I am about to describe, the tubes receive equal illumination and are compared against a dark background. A series of standard suspensions is used.

The method possesses the following advantages.

- (1) It is rapid and reasonably accurate.
- (2) It can be applied to the direct estimation of the number of cells present in growing cultures of yeast, and observations can be made on the cultures while growth is in progress without risk of exposure to infection.
- (3) No expensive or elaborate apparatus is required.
- (4) Although the preparation of a series of standard suspensions takes a considerable time, these standards appear to keep indefinitely. I have in use at the moment a series which was prepared eighteen months ago.

### *Standard suspensions of barium sulphate.*

*General considerations.* The conditions employed in the precipitation of barium sulphate have been shown by Folin [1905] to influence to an important extent the character and composition of the precipitate obtained. In the

preparation of the standard suspensions every effort was made to keep the conditions absolutely uniform, in order to secure uniformity in the precipitates. Moreover, the special conditions recommended by Folin for the precipitation of potassium sulphate solutions have been adhered to as closely as possible.

To avoid the errors which would occur in the measurement and dilution of suspended barium sulphate, each standard suspension was prepared separately.

The test-tubes selected to contain the standards, and those used for the growth of yeast cultures, were of uniform bore.

While the excess of barium in the suspensions was probably adequate to prevent the growth of any organism, mercuric chloride was added as an additional precaution. Denis [1921] has shown that this does not interfere with the precipitation of barium sulphate. The mercuric chloride which I employed gave no precipitate on addition of acidified barium chloride solution.

For the sake of convenience I have considered as a dissolved substance the barium sulphate present in a suspension, and expressed its "concentration" in terms of molarity. If  $m$  be used to express molar concentration  $\times 10^{-5}$ , or  $M/100,000$ , then the concentrations of barium sulphate in the series of standards which I have used are as follows:

1000  $m$ , 900  $m$ , 800  $m$ , 700  $m$ , 600  $m$ , 550  $m$ , 500  $m$ , 450  $m$ , 400  $m$ , 350  $m$ ,  
300  $m$ , 250  $m$ , 200  $m$ , 160  $m$ , 100  $m$ , 80  $m$ , 60  $m$ , 40  $m$ , 20  $m$ , 10  $m$ .

The cloudiness of any suspension containing less than 10  $m$   $\text{BaSO}_4$  is too slight for accurate comparison with yeast suspensions. Concentrations greater than 1000  $m$  have not been employed, as the precipitate settles too rapidly. This difficulty might have been overcome by the addition of ammonium nitrate, as suggested by Denis.

*Method of preparation.* The solutions employed were:

barium chloride, approximately  $M/4$ ,  
mercuric chloride, saturated solution,  
potassium sulphate  $M/40$  and  $M/200$  (Kahlbaum).

From the two solutions of potassium sulphate further dilutions were made, namely,  $M/2000$  from  $M/200$ , and  $M/400$  and  $M/4000$  from  $M/40$ . Each suspension was prepared as follows. To 5 cc. mercuric chloride solution in a 250 cc. flask I added successively 2 cc. pure concentrated hydrochloric acid, the requisite volume of potassium sulphate solution, and sufficient barium chloride solution to give an amount of barium in solution at least equal to that present in the precipitated form. For example, in preparing 200  $m$   $\text{BaSO}_4$  I used 100 cc.  $M/200$  potassium sulphate and added 5 cc. barium chloride. The barium chloride was added drop by drop at the rate of 2.5 cc. per minute, without shaking or moving the mixture in the flask, which was allowed to stand undisturbed for a further five minutes. The flask was then filled to the mark with distilled water, shaken very thoroughly, and a sample was transferred as rapidly as possible to a clean test-tube. The tube was immediately sealed in a flame.



*Apparatus for comparing turbidities of suspensions.*

For comparing turbidities I have used the apparatus shown in Fig. 1. When in use, the hinged lid (*F*) is closed and observation is made through the apertures in it (*A*). As a rack to hold the tubes I have used a modified Cole and Onslow's comparator (*B*) [Cole, 1926] in which the four compartments employed are as close together as possible, and are drilled out through the bottom to allow light to pass up from below. All the inner surfaces of the comparator as well as the apertures at the back of the posterior compartments are covered with matt-surfaced black paper. As a source of light (*C*) I have used a 40-watt gas-filled lamp with opal globe, but owing to the heat it produces I have found it necessary to cool the whole apparatus by means of an air current from an electric fan. To secure approximately equal illumination of the tubes I have inserted between the comparator and the lamp four pieces

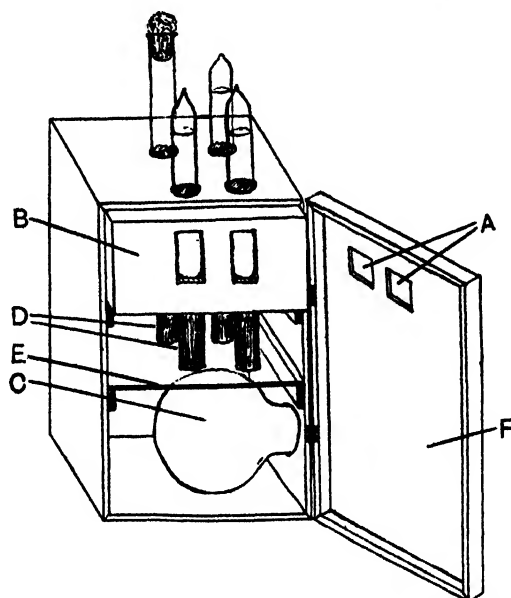


Fig. 1.

of glass tubing (*D*) of uniform length and diameter. These are lined with black paper and rest below on a glass plate (*E*). Above, they are fixed into the compartments of the comparator by means of rubber tubing. The lamp is arranged so that two samples of a suspension of yeast (or barium sulphate) display the same turbidity when compared in the apparatus; this being considered as a sufficiently accurate criterion of equal illumination of the tubes. The apparatus should be used in a dark room, but I have obtained quite good results in daylight by fixing a long tube of cardboard with one end around the windows (*A*), and applying the eye to the other end.

*Estimation of yeast suspensions in coloured and colourless fluids.* In comparing the turbidity of suspensions in colourless fluids I have placed two tubes

of distilled water in the anterior pair of compartments in the comparator, and the tubes containing the suspensions in the posterior pair. This is the arrangement shown in Fig. 1, in which a tube containing a yeast culture is pictured in the left-hand compartment, and a standard in the right. A small degree of colour in the yeast suspension does not interfere with the comparison.

I have had little occasion to estimate suspensions which were coloured to such a degree as might interfere with the estimation. But in the estimation of the most highly coloured suspensions a control tube of the coloured fluid (containing no suspended matter) must be substituted for the tube of water in front of the standard suspension. At the same time a screen must be placed between the source of light and the standard tube to compensate for the cutting off of light from the suspended particles, owing to the colouring matter. This screen should be chosen of such a colour that, on looking down through a tube of distilled water placed over it, and through a control tube of the coloured fluid, the same tint is observed, the tubes being filled to the level of the upper margins of the windows in the front of the comparator. Obviously this procedure does not perfectly control the colour factor, but preliminary experiments have shown that it yields very good results in the estimation of suspensions of yeast in highly coloured fluids.

In all comparisons of turbidity the suspensions were shaken as thoroughly as possible and compared in the apparatus immediately. Growing cultures of the yeast "381" readily form uniform suspensions if the tube is held upright between the thumb and fingers and shaken with a slight rotatory movement. This is especially the case up to the third day of growth. Subsequently, the yeast tends to adhere to the bottom of the tube and some pigment formation commences which renders more difficult the comparison with the barium sulphate standards, particularly in the case of rich development of the yeast. I have frequently observed that up to the third day of growth the cultures contain a very high proportion of single cells, and only a few cell-aggregates containing more than two cells. As growth continues larger aggregates generally predominate, and these tend to adhere to the walls of the tube. By admitting a small glass bead I have been able to obtain suspension of the cultures more easily, and experiments are in progress to determine whether this procedure is injurious to the yeast.

Thus the method is most suitable for the estimation of young growing cultures of yeast and becomes less accurate and convenient as the age of the cultures increases. This does not appear to me to be a great disadvantage as the growth of most cultures is very slow after the 70th hour.

### RESULTS.

The method has been applied to the estimation of suspensions of two species of yeast. Results are tabulated below (Table I) and recorded graphically in Fig. 2. They may be considered as calibrations of the barium sulphate suspensions in terms of yeast.

For each suspension of yeast a figure is given in the third column of the table denoting the concentration of cells it contained, and in the fifth column showing the concentration of barium sulphate which was required to display the same turbidity. In cases where the turbidity was not exactly matched by a standard I have judged the concentration of the standard which would have matched it by reference to the two standards nearest to it. In these cases I have shown the figures for turbidity in parentheses.

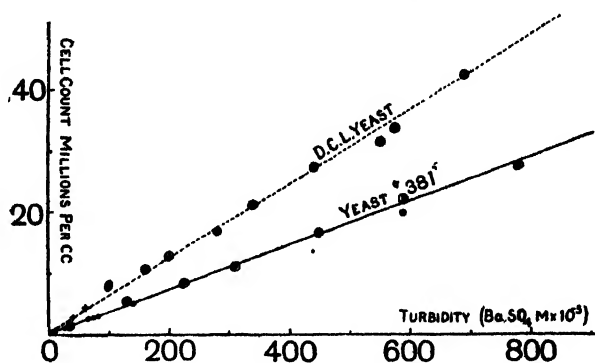


Fig. 2.

The remarks in the fourth column of the table need some explanation. In determining the number of cells present by a count a Bürker chamber was used, four samples were usually counted, and the mean taken. The maximum deviation of the figure for any individual sample from the mean was 21 %, the minimum 1 %, while in most cases the deviation was about 8 %. In the earlier determinations only the large squares of the chamber were counted. Such procedure necessarily limits the concentration of cells which can be determined by a direct count—without dilution—to some value between 0.25 and 5.0 millions per cc. But any suspension containing more than 50,000 cells per cc. possesses turbidity to a degree which can be matched by one of the standard suspensions. Thus in many cases where the turbidity of a yeast suspension was determined its cell content was not actually estimated by a count, but was calculated from the dilution used in preparing it. The remarks in the fourth column of the table refer to the method employed in obtaining the figures recorded in the preceding column. "Observed" values were determined by cell-count, whereas "calculated" values were calculated from the dilution of the suspension which was used.

The counts of suspensions II and III show that the discrepancy involved in diluting suspension II ten times was 14 %. But the counts of the four samples of suspension III showed a deviation of 21 % from the mean, presumably because the concentration of cells was low. In more recent determinations, of which suspensions XXIX and XXX are typical examples, larger concentrations have been counted directly using the small squares in the Bürker chamber. The error in diluting suspension XXIX ten times to

prepare XXX was 4.5 %. Thus when counts have been made of diluted suspensions the results have agreed with the values calculated from the dilution within the limits of experimental error. I therefore feel justified in including in my results those observations in which direct counts were not actually made, but in the graph such observations have been charted with distinguishing marks, i.e. enclosed in small circles ( $\odot$  and  $\oplus$ ).

Table I.

*Fresh "D.C.I." Yeast.*

Suspension number	Method of preparation	Millions of cells per cc. = C	Observed or calculated	Turbidity in terms of $\text{BaSO}_4 M/10^{-5}$ = T	T/C
I	Suspended in water containing phenol	31.5	Calculated (from mean of II and III)	550	17.46
II	25 cc. I in 250 cc.	2.93	Observed	40	13.66
III	50 cc. II in 500 cc.	0.34	"	(4)	11.78
IV	25 cc. I in 100 cc.	7.9	Calculated	100	12.66
V	100 cc. II in 500 cc.	0.59	"	(9)	15.26
VI	Another sample suspended as I	42.2	Calculated (from VII)	(690)	16.37
VII	50 cc. VI in 500 cc.	4.22	Observed	60	14.24
VIII	25 cc. VI in 100 cc.	10.55	Calculated	160	15.18
IX	50 cc. VI in 100 cc.	21.1	"	(340)	16.12
X	30 cc. VI in 100 cc.	12.66	"	200	15.80
XI	40 cc. VI in 100 cc.	16.88	"	(280)	16.60
XII	65 cc. VI in 100 cc.	27.42	"	(440)	16.08
XIII	80 cc. VI in 100 cc.	33.76	"	(575)	17.05
XIV	25 cc. XIII in 100 cc.	8.44	"	100	11.86

Mean = 15.01

*Yeast "381."* This is a pure strain of *S. cerevisiae* which I have used throughout a series of studies on yeast growth. "381" is the catalogue number in the National Collection of Type Cultures. Suspension XV was prepared from a 48 hours' growth at 25°, on medium containing cane sugar, salts and yeast extract. The cells were thoroughly washed on the centrifuge before use.

XV	Suspended in water containing phenol	27.7	Calculated (from XVI)	(780)	28.16
XVI	5 cc. XV in 50 cc.	2.77	Observed	(75)	27.07
XVII	40 cc. XV in 50 cc.	22.16	Calculated	(590)	26.65
XVIII	30 cc. XV in 50 cc.	16.62	"	450	27.09
XIX	10 cc. XV in 50 cc.	5.54	"	(130)	23.46
XX	25 cc. XVI in 50 cc.	1.39	"	(35)	25.20
XXI	5 cc. XX in 50 cc.	0.14	"	(3)	21.43
XXII	25 cc. XVII in 50 cc.	11.08	"	(310)	27.99
XXIII	25 cc. XVIII in 50 cc.	8.31	"	(225)	27.07
XXIV		0.24	Observed	(8)	33.33
XXV	Isolated observations on various cultures	5.0	"	(140)	28.00
XXVI		2.91	"	80	27.50
XXVII		2.68	"	(65)	24.27
XXVIII		0.96	"	(27)	28.14
XXIX	Another sample suspended as XV	19.75	"	(590)	29.96
XXX	5 cc. XXIX in 50 cc.	1.87	"	60	32.07

Mean = 27.34

### DISCUSSION.

If the figures in the third and fifth columns of the table are plotted on a graph, as is shown in Fig. 2, the points fall on a straight line, the slope of which depends on the species of yeast considered. There is a direct relationship, therefore, between the number of cells present per cc. and the "concentration"

of barium sulphate which is required to give the same turbidity. By determining the latter in the case of a given suspension of yeast its cell-content can be estimated by reference to the graph, or by calculation from the constant T/C. If the lines plotted, or the mean value of the constant T/C, can be considered as representing the true relationship between turbidity and number of cells, and the fact that they are derived from a number of observations seems to justify this assumption, then it will be seen that, for the yeast "381," the maximum error of any single observation is 17 %, while over the greater part of the graph the error is not greater than 3 %. For "D.C.L." yeast the corresponding figures are 21 % and less than 7 %. These figures compare favourably with the average error involved in a direct cell-count. I have not considered the error of the figures for suspensions showing a turbidity less than 10 *m* BaSO<sub>4</sub> for reasons already stated.

The graph shows that the method can be used conveniently for the estimation of the concentration of cells in yeast suspensions between 0.5 and 35 millions per cc., in the case of yeast "381." The estimation is less accurate for smaller concentrations, and is not possible if there are less than 40,000 cells per cc. In the case of concentrations greater than 35 millions per cc. the suspension of cells must be diluted to come within the range of the standards.

It would perhaps be simpler to use some suspension other than barium sulphate as a standard, *e.g.* gum mastic. One standard suspension of such a substance could be employed, and the turbidity of the cell suspension determined in terms of the dilution of this standard which would be required to display the same cloudiness. I have not attempted any modifications of this nature, as the method in its present form has quite fulfilled all my requirements. Indeed, I hope that it may be modified for use in the estimation of cells other than yeast, *e.g.* bacteria, blood corpuscles, etc.

#### SUMMARY.

A method is described for the rapid estimation of the number of cells in suspensions of yeast, based on a comparison of the turbidity of the suspension with that of standard suspensions of barium sulphate.

In conclusion I desire to thank Prof. Peters for his advice and criticism, and the Medical Research Council for a part-time grant.

#### REFERENCES.

- Cole (1926). *Practical Physiological Chemistry*. (7th Edit. Heffer and Sons.)
- Denis (1921). *J. Biol. Chem.* **40**, 313.
- Folin (1905). *J. Biol. Chem.* **1**, 131.
- McFarland (1907). *J. Amer. Med. Assoc.* **40**, 1176.
- Richards and Wells (1905). *Amer. Chem. J.* **31**, 235.
- Star (1894). *Œuvres*, **1**, 155.

## LXVIII. ON THE ISOLATION FROM TISSUES OF CERTAIN PENTOSE DERIVATIVES.

By LEWIS BLAND WINTER.

(*Fishmongers' Company Mark Quersted Exhibitioner.*)

*From the Biochemical Laboratory, Cambridge.*

(*Received March 17th, 1927.*)

IN a letter to *Nature* [Winter and Smith, 1923, 1] reference was made to a carbohydrate present in rabbits' tissues which appeared to be of an unusual type. At that time it was believed [Dudley and Marrian, 1923] that an animal at the point of insulin convulsions had little or no glycogen present in the tissues, and it was suggested that there might be a conversion of glycogen or glucose into this unidentified sugar under the action of the insulin. The chief point that was noted about this substance was its apparent instability during the process of isolation from the tissues. Extracts which had been kept for a short time in a semi-dry state showed a markedly diminished sugar content as evidenced by the lessened intensity of the Molisch reaction, and in some cases this test became negative. It was also found that boiling for a short time with very weak acid would appreciably restore the property of giving a positive  $\alpha$ -naphthol reaction. The more recent experiments to be described have confirmed the truth of these observations. Though the fresh extracts were without action on Fehling's solution, before and after hydrolysis with mineral acid, it had been found that the incubation of such an extract with fresh muscle tissue led to an increase in the amount of reducing sugar, suitable control experiments being performed. This indicated that there was in the extracts a carbohydrate which was potentially a reducing sugar, yet combined in such a way that the reducing power was masked; and that hydrolysis with acid was not capable, or had been carried out under such conditions that it was not capable, of liberating the reducing sugar. It is only as a result of later work with the methylated substance that it has been possible to demonstrate definitely the reducing nature of the sugar. At that time there was great difficulty in attempting to isolate such a sugar, since there was only the  $\alpha$ -naphthol reaction that could be used as a guide during the many operations. It was suggested by Berkeley [1923] that the unknown substance was of a pentose nature. Reasons were given [Winter and Smith, 1923, 2] for thinking that this was incorrect, since the extracts used were not sufficiently fresh to give more than minimal amounts of furfural. Later, when it had been recognised that the estimations must be carried out within a short time after extraction, it was found

that considerable quantities of furfural were formed on distillation with 12 % HCl, and since then this has been used as the method for checking the different fractions during the process of isolation. The work has been carried on continuously during the past three years, and although it is still incomplete, it must be interrupted for a time. The following is an account of the results which have so far been obtained.

#### EXPERIMENTAL.

In working with the liver and muscle of rabbits, alcoholic extraction was used. When it was found necessary to work on a large scale, the quantity of fat removed by the alcohol made the subsequent operations very difficult. The use of acetone is a great improvement in this respect. Normal goats were used as the source of fresh liver and muscle. Determinations of the pentose content (unpublished experiments with W. Smith) of the tissues of normal and insulinised animals did not show sufficient increase in the latter to warrant the use of large amounts of insulin, and in many cases the results were inconclusive. It was soon recognised that if the sugar was as unstable as the preliminary experiments had indicated, the greatest chance of success in isolation of the substance lay in stabilising it at an early stage after extraction. A means of alkylating the sugar was therefore looked for. To carry it out effectively it was desirable to precipitate the pentose sugar, as adsorption compound or otherwise, in order to obtain it relatively free from other substances. Though the precipitant adopted, silver, only carries down a part of the desired sugar, to methylate sugar contained in such a large syrupy mass as results from concentration of the acetone extract of material from only one goat was obviously out of the question; and the alternative of attempting to remove other constituents of the syrup, leaving the half gram of pentose, was equally impossible, even supposing that the sugar would not have been destroyed in the large number of necessary operations, and the preliminary experiments all tended to suggest that such destruction would take place. It has been found that, though precipitation with silver is effective in removing a considerable amount of pentose, the sugar cannot again be precipitated with this reagent. There is no difference in this respect whether the silver is removed as sulphide or chloride. After removal of hydrogen sulphide or chloride from the filtrate, followed by concentration of the solution *in vacuo*, the re-addition of silver sulphate causes the formation of a precipitate, which does not contain more than a trace of furaloid substance. This fact suggests that the unstable pentose is held as adsorption compound on the original silver precipitate, which probably consists largely of purine material. At this stage the only evidence of the presence of pentose was the formation of furfural on distillation with acid. Glycuronic acid reacts in this manner, and it was essential to be sure that the latter substance was not responsible for the facts observed. The naphthoresorcinol test was usually negative, even when a large amount of pentose was indicated by the quantitative estimation. On some occasions a

slight positive reaction was obtained, but there was no constancy in the results from this test.

No attempt has been made to keep the extracts of liver and muscle separate, since the sole object of the experiments was the isolation of as much material as possible. It has been found impossible to work separately with the liver of a goat, since the large amount of reducing sugar which is formed in the short time before removal of the organ causes rapid reduction of the silver solution. When the liver is minced and mixed with the muscle, the combined extract can be dealt with without difficulty, and it has therefore been possible to infer that the liver brings to the silver precipitate approximately the same amount of pentose as does the whole of the muscle. Two goats are usually used. They are pithed, bled and skinned; the livers and as much muscle as possible are removed, minced, and placed in cold commercial acetone in the proportion 1.25 l. per kg. of fresh tissue (about 14 kg.). The mixture is allowed to stand with occasional shaking for two days at room temperature. The liquid is then strained off and filtered, and as much acetone as possible removed from the tissue by means of a press. The acetone is then distilled off, and the watery extract concentrated under reduced pressure at 40° until the volume is about 500 cc., the concentration being completed about nine hours after the commencement of filtering. The thin syrup is thoroughly cooled, and treated with saturated baryta (cold) until no further precipitation occurs; this point is readily observed with experience owing to the flocking of the precipitate which takes place. The fluid is then centrifuged, and the clear solution poured off. The precipitate is stirred up very thoroughly with cold water. After centrifuging, the washings are added to the main bulk, and the whole neutralised with dilute sulphuric acid. Using a large machine of 1200 cc. capacity, the liquid need not be alkaline for more than 30 minutes; it is unlikely that in this time the alkalinity will have done more than bring about equilibrium between stereoisomers, certainly no appreciable resinification of sugars occurs. It has not been found possible to avoid the use of baryta. The barium sulphate is not removed from the neutralised fluid, but excess of silver sulphate is added. Approximately 6 l. of the saturated solution are required for about 14 kg. tissue, and when the precipitate flocks and begins to settle appreciably within two or three minutes after the last addition, the right point is reached. The mixture is then placed in the dark for an hour, and as much as possible of the clear fluid is siphoned off. The remainder, usually about 3 l., is centrifuged. It is important to protect the material from the action of light at this stage. The solid in each cup is stirred up with 95 % alcohol, and again spun down. Then follow two washings with absolute alcohol, and two with dry ether. It was originally decided to methylate the sugar contained in the silver precipitate with methyl iodide and silver oxide, as in the methods developed by Purdie and Irvine; the last ether washing was therefore poured off, and the solid transferred to a flask. 100 cc. dry methyl alcohol, and 50 g. methyl iodide were added, and the mixture was heated at 50–60° for two hours.



After the addition of 10 g. silver oxide, and 10 g. methyl iodide, heating was maintained for a further four hours. The silver was filtered off and washed with methyl alcohol; the filtrate was evaporated to dryness, and heated at 70–80° *in vacuo* overnight. Next day about 20 cc. cold methyl alcohol were necessary to dissolve the syrup; 20 g. methyl iodide were added, followed by 10 g. silver oxide, in small amounts. For the third methylation 20 g. methyl iodide were used, and the syrup was partly soluble in this medium, only 2 cc. methyl alcohol being necessary to take the whole into solution. 16 g. silver oxide were added slowly, followed later by 20 g. methyl iodide, and 8 g. silver oxide. Two further alkylations were performed using methyl iodide alone. The syrup finally obtained was distilled at 13 mm. pressure. The first fraction began to come over at 110°, and the temperature continued to rise to 180°, with a bath-temperature that was gradually increased to 240°. Three fractions were collected. Considerable charring took place during distillation. The distillates were soluble in water and ether, and gave a Molisch reaction. After hydrolysis with acid a reducing substance was present in the solution. It is remarkable that the distillates all contained organic phosphorus, the amount increasing with the boiling-point. This result was confirmed in all cases in which the methylation was carried out in the above manner. Though the nature of the phosphorus-containing substance in the distillates has not been established, it is very probable that it is derived from a pentose-phosphoric acid compound, such as inosinic acid which is very likely to be present in the silver precipitate. The phosphorus is only slowly liberated on hydrolysis with acid.

0.3 g. of the fraction boiling between 115° and 130° was heated on the boiling water-bath with 4 cc. of *N* sulphuric acid.

Time (mins.)	0	10	20	65
<i>a</i>	+ 1.72°	+ 1.12°	+ 1.15°	+ 1.15°

The acid was removed as barium sulphate, and the filtrate was concentrated to dryness. The syrup was extracted with ether, and distilled after removal of the solvent. The distillate reduced Fehling's solution vigorously, and contained organic phosphorus in considerable amount. As it was unlikely that the sugar sought was in combination with phosphorus, for the pentose of inosinic acid is stable and not likely to show the same phenomenon of a diminished capacity to form furfural when kept for any length of time, a method of removing the phosphorus compound was desirable. This was found to be very difficult to effect by fractional distillation, and, as none of the distillates showed signs of crystallising, the only solution lay in preventing the phosphorus compound from being methylated at the same time as the other pentose. Preliminary extraction of the silver precipitate with methyl alcohol failed to remove the phosphorus-containing substance, but the use of methyl sulphate, as in Haworth's method of alkylating sugars, proved successful in keeping back the phosphoric acid from the outset. The silver precipitate was suspended in dry ether in a dark bottle and decomposed by passing dry HCl gas through

it. This stage was always reached on the same day as the acetone extract was filtered off. Twelve hours later the ether was removed, and the solid shaken with a further quantity of ether (about 100 cc.). After standing for a further 12 hours the ether was replaced with 75 cc. dry methyl alcohol, and the precipitate allowed to stand in contact with the alcohol for two days at room temperature with frequent shaking. The alcohol was then removed and the precipitate shaken with a further 75 cc. of alcohol. Meanwhile the first extract was being shaken with silver carbonate. The second alcoholic solution was separated after 24 hours, added to the first, and the last traces of acid removed. After this treatment a water extract of the silver residues showed only traces of pentose remaining. The pentose content of the alcohol extract was estimated as a routine procedure by removing a known small amount, distilling with hydrochloric acid, and weighing the precipitate of phloroglucide. The solvent alcohol was first removed on the water-bath. In order to methylate the sugar contained in the alcoholic extract with methyl sulphate it was necessary to remove almost the whole of the alcohol, and, whilst it is possible that some destruction of the furaloid substance might occur during concentration, the test samples were treated in a similar manner and would indicate the amount of pentose which was available at the time of methylation. Up to 0.8 g. pentose has been shown in the extract from two goats; sometimes it has been much less than this. For a considerable time it was customary to keep the methyl alcohol solution, freed from acid, of each pair of animals at 0°, until four such extracts were available, and then to combine them and concentrate for methylation. It was assumed that the pentose would be stable under these conditions. The four combined extracts were then concentrated under reduced pressure, or directly on the water-bath (no apparent difference in the yield being observed in this respect), down to 3–4 cc. The syrup was then transferred to an apparatus fitted with mechanical stirrer and dropping funnels, and treated with methyl sulphate and sodium hydroxide. A very vigorous reaction ensued, and the temperature was not allowed to rise above 50° until half the necessary quantity of the reagents had been added, in successive small amounts. For this amount of extract 30 cc. methyl sulphate and 48 cc. sodium hydroxide solution (40 g. in 85 cc. water) were used. The whole of the reagents having been added, the bath-temperature was raised to 70° and maintained at this level for an hour. After boiling for half an hour, the contents were cooled, and extracted twice with chloroform. The chloroform extracts were dried by standing over anhydrous magnesium sulphate for several days, filtered into a flask and the solvent removed. Methylation was then continued with methyl iodide and silver oxide, the whole of the syrup being soluble in the iodide. After heating for six hours, the silver residues were extracted with ether; the solution was dried, the solvent removed, and the syrup distilled under 13 mm. pressure. 0.6 g. was collected boiling between 125° and 131° and, on changing the receiver, no further distillate was collected when the temperature of the bath was raised. A considerable amount of non-volatile

residue remained in the flask. The distillate was neutral and entirely free from phosphorus, while an intense Molisch reaction was obtained. After heating a sample with *N* sulphuric acid at 100° for an hour, the solution reduced Fehling's solution. The next four pairs which were dealt with in a similar manner gave a very poor yield of the methylated derivative, only 0.2 g. being obtained. The initial pentose content of the two batches as deduced from the test sample taken from each alcohol extract was very similar, and it became evident that there was some secondary change proceeding which must account for the wide variation in the yield.

*Examination of the alcoholic solution before methylation.*

As evidence was accumulating as a result of the alkylation experiments that the pentose substance when methylated was capable of reducing an alkaline copper solution after hydrolysis, it seemed desirable to test the solution in methyl alcohol for the presence of a potentially reducing sugar, and to ascertain whether the process of methylation was after all necessary for the isolation of the pentose. It was possible that a reducing sugar might have been liberated during the decomposition of the silver precipitate with HCl in ether, which would condense with the solvent during the extraction with methyl alcohol under the influence of the HCl which was present before removal of the acid with silver carbonate. An extract was used which showed a pentose content of 0.51 g. The alcohol was removed under reduced pressure, and the residue dried in a desiccator for one day. The syrupy mass was then extracted with cold methyl alcohol (30 cc.). A white solid was left which on examination was found to be hypoxanthine. No pentose residue was found insoluble in cold methyl alcohol. The solution was cleared with charcoal in the cold, filtered, and evaporated again in the desiccator. More solid separated and was removed by extraction as before with alcohol, the syrup going into solution very readily. A test sample showed a pentose content of only 0.170 g., and since the residues had been quantitatively examined each time for pentose with negative results, it must be concluded that decomposition of the desired substance had taken place or some alteration which prevented the formation of furfural on distillation with acid. It was clear, therefore, that if the pentose underwent such a profound change during the mild conditions of the above experiments, it was improbable that the sugar could be isolated without recourse to the stabilisation conferred by methylation. The solution was again evaporated; three weeks later the pentose content had not appreciably altered. It is likely that the residual pentose was ribose, and in combination with phosphoric acid and hypoxanthine. There was organic phosphorus present, and hypoxanthine had also been separated from the mixture. Since silver precipitates purines, it was to be expected that such substances would be present. The syrup was taken up in *N* sulphuric acid, and heated at 100°. There was a steady fall in the polarimeter readings, and a very slight reduction of Fehling's solution was observed.

Time (mins.)	0	10	20	30	90
$\alpha$	0-40°	- 0-31°	- 0-19°	- 0-17°	- 0-15°

The silver precipitate from another goat was dried with alcohol and ether and decomposed in the usual manner. During the second extraction with ether, the HCl was removed with silver carbonate. The precipitate was shaken for one hour with a small amount of methyl alcohol, and then filtered. To a part of the clear solution was added some HCl in dry methyl alcohol so that the concentration of acid was 1 %. The optical activity was observed at once, and at varying intervals during the course of 24 hours, but was unaltered. No apparent change was taking place in the carbohydrate under the action of the acid and alcohol, at room temperature. The methyl alcohol extracts are laevo-rotatory, and the methylated derivative finally obtained is dextro-rotatory, the positive value due to the sugar being evidently masked by a large amount of impurity; yet any change in the sugar due to condensation with the solvent should be observed in a change in the optical activity of the mixture. A silver precipitate was dried with alcohol and ether, and then extracted for 5 minutes with methyl alcohol containing 2 % HCl. The solution was centrifuged off and examined, but no change in the rotation was observed on standing. The result was therefore the same whether the sugar was subjected to the action of hydrochloric acid in ether or in methyl alcohol. No evidence was obtained of condensation of the sugar with the alcohol, and if it does proceed it must be completed very rapidly. It was impossible to examine the solution immediately, since a short time must be allowed for some extraction of the precipitate, and the solution has then to be cleared by filtering or by the centrifuge.

The instability of the pentose in the alcoholic extract led to a reconsideration of the advisability of leaving the extract from each pair of animals in cold storage until three or four such solutions were available. It was not possible to deal with more than two goats in one week, the extraction with methyl alcohol not being complete until the evening of the 7th day after the animals were killed. By the end of 3 or 4 weeks the first sample might have a considerably diminished pentose content, even though the mixture was maintained at 0°. This was found to be the case, though the difference was not large as determined by the furfural estimation. When the distillation of the final methylated derivative came to be carried out, however, the difference was remarkable, both qualitatively and quantitatively. While only traces had before been obtained of distillate boiling under 124-125°, now the main fraction was over when this temperature was reached; also, provided that a reasonable amount of pentose had been indicated in the small test samples, a proportional yield of the final product was obtained. In the previous series of experiments there was no certainty that this would be so. Each methyl alcohol extract is now methylated with Haworth's reagents as soon as possible. Directly the acid has been removed, the solution is filtered and concentrated *in vacuo* to a small volume, and treated with 20 cc. methyl sulphate and 32 cc. sodium hydroxide. The united chloroform extracts are kept in the cold store

over anhydrous magnesium sulphate until a number are available. Though not fully methylated, the sugar must be sufficiently stabilised by this time, and there is no advantage in carrying out the alkylation with Purdie's reagents on individual extracts. Three such chloroform extracts were concentrated under reduced pressure, and on the addition of 25 g. methyl iodide the whole of the syrup went into solution. 10 g. silver oxide were added gradually, and the mixture was heated at 55–60° for 7 hours. The silver residues were extracted three times with ether. The ether extract was dried, and some weeks later combined with that obtained in a similar manner from three other pairs of goats. The solvent was removed, and the residue distilled at the water-pump. 1.5 g. of a colourless liquid was collected, boiling between 118° and 122° (fraction 1). The remainder (0.7 g.) distilled at 125–135° (fraction 2). There was a dark viscous syrup left in the flask, which was extracted with ether. Several of these extracts have since been combined, and the contained syrup distilled under a high vacuum. A crystalline fraction was obtained, but these distillates have not yet been examined. The fractions referred to as 1 and 2 were neutral, soluble in water and ether, and gave an intense Molisch reaction. Nitrogen and phosphorus were absent. Kept for a year they have shown no sign of crystallising. Fraction 1 showed  $[\alpha]_{\text{Hg green}} = +10.85^\circ$  in water,  $c = 1.94$ . OMe = 53.51 %. Hydrolysis was not effected by boiling with *N*/100 acid. *N*/10 sulphuric acid slowly liberated reducing sugar. The following observations were made on a very dilute solution (58 mg. in 4 cc. *N*/10 acid).

$\alpha =$	+ 0.14°	0.16°	0.16°	0.16°	0.14°	0.25°	0.24°	0.24°
Time (hours)	0	$\frac{1}{2}$	1	3	6	12	20	28

With normal acid, hydrolysis was rapid, as will be shown later. Fraction 2 showed  $[\alpha]_{\text{Hg green}} = +19.00^\circ$  in water,  $c = 1.53$ . OMe = 52.52 %. This fraction was more readily hydrolysed with *N*/10 acid, and the change in the polarimeter readings is in the opposite direction to that shown by fraction 1. The solution contained 45 mg. in 4 cc. acid.

$\alpha =$	+ 0.16°	0.10°	0.08°	0.07°	0.08°	0.09°
Time (hours)	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$

At the end of this time the solution reduced Fehling's solution markedly. To undertake the methylation of the small quantities of material that were now being dealt with, it was necessary to devise special apparatus. This was constructed by Messrs Flaig and Sons, 57, Hatton Garden, E.C. It consists of a short boiling tube,  $3\frac{1}{2} \times \frac{3}{4}$  ins., ground at the neck to receive a condenser or a bent tube. The boiling tube is slightly expanded at the top, so that when the condenser or distilling tube is fitted into the ground joint, mercury can be poured on to the joint to act as a seal. The condenser is of the double surface type, and measures only  $5\frac{1}{2} \times \frac{7}{8}$  ins. Fraction 1 was transferred to the boiling tube and methylated with 13.5 g. methyl iodide, and 9 g. silver oxide for 7 hours at 50–55°. The silver residues were extracted with ether. The solution was dried and filtered. After removal of the ether, which is accompanied by

unavoidable loss on account of the volatility of the methylated substance, the syrup was distilled at 13 mm. pressure. The distillate was remethylated, using the same quantities of the reagents as before, and again distilled. The distillate was collected in three fractions. The main (middle) fraction was analysed<sup>1</sup>.

3.505 mg. gave 15.552 mg. AgI: 56.00 % OMe.  
 3.599 mg. gave 15.145 mg. AgI: 55.72 % OMe.  
 0.0780 g. gave 0.1481 g. CO<sub>2</sub>, 0.0615 g. H<sub>2</sub>O: C, 51.78; H, 8.76.  
 0.0877 g. gave 0.1669 g. CO<sub>2</sub>, 0.0700 g. H<sub>2</sub>O: C, 51.85; H, 8.86.  
 C<sub>6</sub>H<sub>6</sub>O(OCH<sub>3</sub>)<sub>4</sub> requires C, 52.42; H, 8.74.

A cryoscopic determination of the molecular weight in water gave a value of 211. The specific rotation in water for mercury green light was +17.6° (*c* = 1.81).

0.6 g. of the methylated derivative obtained in a similar manner from eight animals was hydrolysed with 20 cc. of *N* sulphuric acid at 100°.

Time (hours)	0	$\frac{1}{2}$	1 $\frac{1}{2}$	2 $\frac{1}{2}$
$\alpha =$	+ 0.49°	0.53°	0.70°	0.70°
$[\alpha]_{\text{Hg green}}$	= + 16.33°	17.66°	23.33°	23.33°

The acid was removed with barium carbonate, and the solution filtered and concentrated under reduced pressure. The residue was extracted with absolute ethyl alcohol, and the solvent removed by distillation. A viscid acid syrup was left and decomposition occurred on attempting to distil it at 13 mm. pressure. Only a drop of distillate was obtained which was dextro-rotatory, gave the Molisch reaction, and reduced Fehling's solution. This failure to separate the reducing sugar from the products of hydrolysis was not unexpected in view of the results obtained by Carruthers and Hirst [1922] with trimethyl-methyl-xyloside. Apparently it is necessary to start with the pure  $\alpha$ - or  $\beta$ -compound; when a mixture is present a large proportion of breakdown products is formed during the heating with acid. Though in the above experiment the acid used was as weak as possible, and the hydrolysis was stopped as soon as the rotation had ceased to alter, it is possible that the failure to isolate the reducing sugar was due to the presence of two stereo-isomers in the original liquid.

The nature of the parent sugar and of the methylated derivative is as yet uncertain. That the latter is a stable type is clear from the strength of acid necessary to bring about hydrolysis. It is hydrolysed at a similar rate to trimethyl- $\beta$ -methyl-arabinoside. The following figures were obtained with a solution of the latter compound in *N* sulphuric acid.

Time (hours)	0	$\frac{1}{2}$	1 $\frac{1}{2}$	2 $\frac{1}{2}$
$\alpha =$	+ 0.24°	0.31°	0.35°	0.34°
$[\alpha]_{\text{Hg green}}$	= + 52.9°	68.5°	77.8°	74.9°

The hydrolysis is complete in the same time in both cases. Though the rather low results obtained in the methoxyl determinations suggest that the substance

<sup>1</sup> Methoxyl determinations were made by the Pregl micro-Zeisel method; for valuable advice on this and other analyses I am indebted to Mr H. I. Coombs. The values for C and H were determined by Mr F. C. Hall, of the Dyson Perrins Laboratory, Oxford.

is not completely methylated, this is not sufficient to account for the large difference between the specific rotations of the unknown sugar and trimethyl-methyl-arabinoside. Xylose can be excluded at once. Methylated derivatives of lyxose and ribose have apparently not been prepared. Even if future work results in the identification of the methylated sugar from the silver precipitate with an alkylated riboside or lyxoside, there remains the problem of the labile nature of the sugar before methylation.

*Isolation of a derivative of d-ribose.*

The ether extracts of the silver precipitates were examined for pentose at an early stage of the work, and it was found that a small amount of furfural was obtained on distilling the residue after evaporation of the ether. When such a syrup was kept for some months there was found to be no disappearance of pentose as occurred in the methyl alcohol extract. It seemed possible, therefore, that the ether had extracted a small amount of another pentose derivative. The substance was finally isolated from each extract as follows. The acid having been removed from the ether with silver carbonate, the solution was dried with potassium carbonate, followed by sodium sulphate. The filtered solution was distilled slowly through a fractionating column, until the volume of ether was reduced to about 10 cc. 50 cc. of light petroleum were added, and distillation was continued. It is important that the petroleum should have previously been distilled. Only that fraction is used which will come through the column on the boiling water-bath. When the flask contains only 5–10 cc. of fluid, it is removed from the bath, and the liquid at once poured out while still hot. On cooling, fat begins to separate. In the flask remains a very small amount of a semi-solid yellow substance from which, however, nearly the whole of the fat has been removed by the treatment with petroleum. The residue in the flask is taken up in a few cc. of cold absolute ethyl alcohol, in which it is very soluble, and filtered. The solution is evaporated in a desiccator, and the resulting syrup crystallises readily. The crystals are dissolved in water, and the solution is cleared with charcoal to remove the last traces of fatty matter. After evaporating to dryness in a desiccator, the substance is recrystallised from absolute alcohol. The slightly hygroscopic compound was dried *in vacuo* over phosphorus pentoxide until the weight was constant. The pentose content was estimated in the usual manner.

9.6 mg. gave 6.6 mg. phloroglucide. 8.0 mg. gave 5.2 mg. phloroglucide. Using for comparison a pure sample of  $\beta$ -methyl-arabinoside, 10.2 mg. gave 6.9 mg. phloroglucide.

The substance contains only carbon, hydrogen, and oxygen; it does not reduce Fehling's solution until after hydrolysis with acid. Heated in a capillary tube, it begins to soften at 58°, and melts at 65–66° (uncorr.). The specific rotation was determined in absolute alcohol. 0.1634 g. in 4.926 g. alcohol gave  $\alpha = -3.61^\circ$  ( $c = 3.31$ ,  $l = 1$ ).  $[\alpha]_{\text{H}_g \text{ green}} = -108.8^\circ$ . The solubility in water and alcohol and the liberation of reducing sugar on hydrolysis suggest that

the substance is an alkyl-pentoside. Owing to the hygroscopic nature of the compound accurate determination of methoxyl was not possible; a value of 12.27 % OMe was obtained, an approximation which gives support to the suggested nature of the substance. The first step in the identification of the pentose was the preparation of the phenylosazone. A small amount of the pentoside was hydrolysed with 2 % sulphuric acid for 1½ hours on the boiling water-bath. Phenylhydrazine hydrochloride and sodium acetate were added, and the mixture heated at 100° for 20 minutes. The crystals deposited on cooling were recrystallised from dilute pyridine. The specific rotation for mercury green was  $-70.2^\circ$ , Neuberg's pyridine-alcohol mixture being used. The crystalline form of the osazone was identical with that prepared from a sample of arabinose, and the sugar is therefore *d*-arabinose or *d*-ribose. A sample was then hydrolysed, and the optical changes recorded. 0.1408 g. in 10.27 g. 2 % sulphuric acid was heated under reflux on the boiling water-bath.

Time (mins.)	0	45	65	85	135
$\alpha =$	$-1.19^\circ$	$0.56^\circ$	$0.40^\circ$	$0.27^\circ$	$0.29^\circ$
$[\alpha]_{H_g \text{ green}} =$	$-86.8^\circ$	$40.9^\circ$	$29.4^\circ$	$19.7^\circ$	$21.2^\circ$

The final specific rotation is very nearly that of *d*-ribose ( $[\alpha]_D - 19.0$ ), and the identity of the sugar is established. It is uncertain whether the crystalline substance is a methyl- or ethyl-riboside, for there was only a small quantity available for investigation. The yield of the pure substance is only about 2.5 mg. per kg. fresh tissue. If the riboside does not pre-exist as such in the tissues, but is formed from ribose set free from inosinic or guanylic acids during the extraction with ether, it seems likely that it will be the ethyl compound due to reaction with traces of ethyl alcohol, persisting from the operation of drying the silver precipitate, under the catalytic influence of the hydrochloric acid. On the other hand, it is to be expected that a mixture of the  $\alpha$ - and  $\beta$ -forms would be produced under these conditions, and the complete crystallisation which takes place when the solution in absolute alcohol is evaporated suggests that only one isomer is present, from the optical activity probably the  $\alpha$ -riboside. The curve of polarimeter readings on hydrolysis is regular and indicates the absence of the  $\beta$ -compound, assuming the difference usually found in the rates of hydrolysis of  $\alpha$ - and  $\beta$ -sugar derivatives. There is no evidence then for the existence of both isomers in the crystalline material obtained, and it might be that the more soluble form was left behind in the light petroleum used for precipitating the substance. Examination of this solution, however, showed no trace of pentose. Using arabinose, no formation of an ethyl-arabinoside was observed when a suspension of the sugar was treated in a similar manner to the silver precipitate. 200 mg. arabinose were shaken with 200 cc. dry ether, containing 2 cc. absolute alcohol, and 2 % HCl, for 24 hours. The sugar was apparently unchanged, and no weighable residue was obtained on evaporating the ether after removal of acid. It seems unlikely, therefore, that the riboside which has been isolated is artificially produced from nuclear material during the processes used for its extraction, and



it is probable that it is present in the fresh tissues. The pentose which was isolated in the form of its methylated derivative is present in relatively large amount as compared with the alkyl-riboside, approximately 0.005 g. as compared with 0.0002 g. per 100 g. fresh tissue. Actually, however, the quantity is very small, and this fact in conjunction with the marked lability of the compound renders it probable that the sugar is an intermediate in carbohydrate metabolism.

#### SUMMARY.

Results are given of an investigation of two pentose derivatives present in animal tissues. One contains *d*-ribose, and is probably an alkyl-riboside. The other is unstable, and only the methylated derivative has been isolated. Its nature has not been determined.

I wish to thank Sir F. G. Hopkins for his continued interest throughout the work and for much expensive material used. For the year 1925 the remaining expenses were partly defrayed by a grant from the Government Grant Committee of the Royal Society. This research was contained in reports to the Trustees of the Beit Memorial Fellowships for Medical Research in the years 1924-26.

#### REFERENCES.

- Berkeley (1923). *Nature*, **112**, 724.  
Carruthers and Hirst (1922) *J. Chem. Soc.* **121**, 2299.  
Dudley and Marrian (1923). *Biochem. J.* **17**, 435.  
Winter and Smith (1923, 1). *Nature*, **111**, 810.  
—— — (1923, 2), *Nature*, **112**, 829.

# LXIX. THE ETHER-SOLUBLE SUBSTANCES OF CABBAGE LEAF CYTOPLASM.

## III. THE FATTY ACIDS.

By ALBERT CHARLES CHIBNALL  
AND HAROLD JOHN CHANNON (*Beit Memorial Research Fellow*).

*From the Department of Physiology and Biochemistry,  
University College, London.*

(Received March 31st, 1927.)

A METHOD of preparing the ether extract of leaf cell cytoplasm in bulk from green unheaded cabbage (*Brassica oleracea*) has been described in a previous paper [Chibnall and Channon, 1927, 1]. A table showing some of the fat constants of the material was given, and it was pointed out that the addition of 4 volumes of acetone to the ether extract results in the precipitation of about 40 % of the material. The chemical nature of certain of the constituents of this precipitate has already been discussed [Chibnall and Channon, 1927, 2] and further investigations of the remaining substances present in it are in progress. The fraction of the extract which is not precipitated from ether by acetone—some 60 % of the whole—contains the fatty acids which are not in complex combination in phosphorus-containing substances. It is with these fatty acids that this communication deals.

On evaporation of the ether-acetone extract *in vacuo*, there is obtained a hard, waxy mass which is almost black in colour on account of the presence in it of chlorophyll. It would obviously have been desirable to remove the pigment before proceeding further, but as this was impossible without the loss or chemical alteration of some of the other substances present, it was necessary to use this pigmented material without further treatment.

### *Preparation of the fatty acids and the unsaponifiable matter.*

After removal of the ether and acetone *in vacuo*, the material was saponified with excess of strong alcoholic potash for 1 hour, and the alcoholic solution poured into water. From this aqueous solution the unsaponifiable matter was removed by exhaustive extraction with ether and the ether extract washed with water. The water washings were united to the main bulk of the aqueous soap solution. On evaporation of the ether solution, there remained a hard orange brown mass which was re-saponified with 2 N sodium ethylate, and then extracted as before. The aqueous solution and the washings from the

ether extract were united to the main bulk of the soap solution obtained during the initial saponification. On removal of the ether, there was obtained the unsaponifiable fraction, the nature of which will be discussed in a subsequent communication.

The aqueous solution, after the removal of the unsaponifiable matter, will contain not only the soaps produced by the saponification of all the esters present in the free fat, but also the alkali salts of the acids produced by the degradation of the chlorophyll originally present. On acidification of the soap solution therefore, there will be precipitated not only the fatty acids but also the chlorophyll derivatives, which Willstätter's researches [Willstätter and Stoll, 1913] indicate will be phytychlorin *e*,  $C_{32}H_{32}ON_4(COOH)_2$  and phytorhodin *g*,  $C_{32}H_{30}O_2N_4(COOH)_2$ . Ether extraction of the soap solution removes the fatty acids and these "chlorophyll" acids. After the usual treatment of the ether extract, there remains behind a black fatty mass which can be fractionated into products soluble and insoluble in light petroleum.

The former are the fatty acids whilst the latter are the chlorophyll derivatives with, possibly, hydroxylated acids. The following table illustrates the general nature of the unsaponifiable matter and the fatty acids, obtained by the foregoing methods from the ether-acetone filtrate.

Table I.

Batch	Autumn sown (picked April and May)				Spring sown (picked August)	
	<i>D</i>	<i>E</i>	<i>F</i>	<i>G</i>	<i>K</i>	<i>M</i>
% of total ether extract not precipitated by acetone	59.5	58.0	59.0	58.5	56.0	60.1
Iodine value	141	147	152	143	154	—
Saponification value	151	158	152	—	149	—
% of unsaponifiable matter	16.9	16.5	17.0	17.1	24.1	25.0
% sterol	3.63	2.65	3.70	3.61	3.80	3.70
Iodine value	113	117	119	122	105	97
% fatty acids	23.2	23.1	23.0	23.6	15.6	16.3
Iodine value	173.5	203.5	201	197	206	—
Neutralisation value	209	206	198	—	197	—

From this table, it will be seen that the general nature of the ether extract in all the batches is very similar save that there appears to be a definite difference in the amounts of the fatty acids and of the unsaponifiable matter in the material obtained during the spring and summer.

*Fatty acids soluble in light petroleum.*

By evaporation of the solvent, the fatty acids remain as a pale yellow, almost liquid mass, free from chlorophyll degradation products.

*Saturated acids.* 7.8 g. of batch *H* and 5.7 g. of batch *E*, 13.5 g. of fatty acids in all, having an iodine value of 205, were treated by the method of Levene and Rolf [1922]. By fractionation of the barium soaps in benzene containing 5 % of 95 % alcohol, there were obtained 4.63 g. of more saturated

acids having an iodine value of 163, 7.87 g. of less saturated acids having an iodine value of 218. The result of this treatment of the barium soaps shows that the separation has not been very satisfactory. This is not surprising when it is known that by far the greater bulk of the fatty acids consists of acids of 18 carbon atoms containing 2 and 3 double bonds; the precipitate of the barium soaps of the saturated acids and oleic acid contains, therefore, a considerable amount of the barium soaps of these unsaturated acids. The 4.63 g. of more saturated acids were further fractionated by conversion into the lead soaps and treatment of these soaps with ether. The soaps insoluble in ether yielded 1.45 g. of solid acid, which was separated into three fractions by crystallisation from 95 % alcohol. These fractions weighed 0.38 g., 0.65 g. and 0.34 g. respectively, and they gave the following analyses:

Fraction (1)	0.0854 g. substance	0.2356 g. CO <sub>2</sub> and 0.0969 g. H <sub>2</sub> O
	0.3772 g. "	required 1.95 cc. $\frac{1}{N}$ KOH for neutralisation
(2)	0.0822 g. "	0.2266 g. CO <sub>2</sub> and 0.0914 g. H <sub>2</sub> O
	0.4802 g. "	required 2.56 cc. $\frac{1}{N}$ KOH for neutralisation
(3)	0.3402 g. "	1.79 cc. $\frac{1}{N}$ KOH "
Calculated	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub> : C, 74.92; H, 12.58.	M.W. 256; M.P. 63.4°
	C <sub>18</sub> H <sub>36</sub> O <sub>2</sub> : C, 75.93; H, 12.78.	M.W. 284; M.P. 70-71°
Found. Fraction (1)	C, 75.23; H, 12.60.	M.W. 271; M.P. 54-55°
(2)	C, 75.18; H, 12.43.	M.W. 263; M.P. 54-55°
(3)		M.W. 266

The amount of material available was obviously too small for the definite identification of the saturated acids present, but the results would suggest that they consist of about 70 % palmitic together with 30 % of stearic acid, the melting-point of which mixture is given as 56° [Lewkowitch, 1921]. It will be seen that 13.5 g. of the acids have given 1.45 g. of saturated acid.

*Unsaturated acids.* The liquid acids generated from the lead soaps soluble in ether were again fractionated by the barium soap-benzene method. The insoluble barium soaps yielded only 0.225 g. of fatty acid having an iodine value of 127. As oleic acid has an iodine value of only 90, it is clear that only a part of this 0.225 g. can be oleic acid; indeed, having regard to the fact that the bulk of the fatty acids are highly unsaturated, it might well be that this 0.225 g. consists not of oleic acid but of saturated acid contaminated by those unsaturated acids.

The 9.2 g. of less saturated acids obtained from the two fractions of the barium soaps soluble in benzene-ether were brominated in anhydrous ether at 0° and were then separated into three fractions: (1) 4.95 g., insoluble in ether, (2) 10.0 g., insoluble in light petroleum, (3) 3.22 g., soluble in light petroleum. These fractions gave the following analyses:

Fraction (1)	0.0879 g. substance	0.0866 g. CO <sub>2</sub> and 0.0301 g. H <sub>2</sub> O
	0.1244 g. "	required (Stepanoff) 10.1 cc. $N/10$ thiocyanate
(2)	0.4133 g. "	" " 28.1 cc. " "
(3)	0.1458 g. "	" " 8.2 cc. " "
Calculated	C <sub>18</sub> H <sub>30</sub> O <sub>2</sub> Br <sub>2</sub> : C, 28.49; H, 3.99; Br, 63.26; M.P. 180-181°	
	C <sub>18</sub> H <sub>30</sub> O <sub>2</sub> Br <sub>4</sub> : Br, 53.28; M.P. 113-114°	
	C <sub>18</sub> H <sub>34</sub> OBr <sub>2</sub> : Br, 36.2	
Found. Fraction (1)	C, 28.12; H, 3.88; Br, 63.02; M.P. 180°	
(2)	Br, 54.30	
(3)	Br, 44.9	

Fraction (2) was debrominated and then hydrogenated with platinum black by the Fokin-Willstätter method. The saturated acids so obtained were crystallised twice from 90 % alcohol and four times from acetone, and gave the following analyses:

0.0850 g. substance	0.2362 g. $\text{CO}_2$ and 0.0991 g. $\text{H}_2\text{O}$
0.5698 g. "	required 20.2 cc. $N/10$ NaOH for neutralisation
Calculated $\text{C}_{18}\text{H}_{36}\text{O}_2$ :	C, 75.93; H, 12.78; m.w. 284; m.p. 70–71°
Found	C, 75.79; H, 12.95; m.w. 281; m.p. 69–70°

Fraction (1) is clearly hexabromostearic acid, showing that the original fat contained 1.78 g. of linolenic acid. Fraction (2) consists of the tetrabromoacids of the  $\text{C}_{18}$  series. Repeated attempts to obtain crystalline tetrabromostearic acid from a variety of solvents failed, and it seems probable that there was present in the original fat a doubly unsaturated  $\text{C}_{18}$  acid which does not yield a solid tetrabromide. The bromine content of fraction (3), namely 44.9 % bromine, suggests that this is a mixture of tetra- and dibromostearic acids of bromine content 53.28 % and 36.2 % respectively. If this be so, then this fraction contains dibromide corresponding to 1 g. of oleic acid, which did not precipitate when the barium soaps were treated with the benzene-ether mixture, possibly on account of the presence of a preponderance of more unsaturated acids; alternatively, all the soaps of the saturated fatty acids may not have been precipitated, and in this case fraction (3) may consist of tetrabromostearic acid contaminated with saturated acids.

*Glycerol.* It was of interest to see whether glycerol was present in the ether extract in a form other than the combination with fatty acids and phosphorus already described [Chibnall and Channon, 1927, 2]. Accordingly the glycerol content of the acetone-ether extract was determined by the method of Zeisel and Fanto [1903]. The flask containing the material was heated to 112° for 2 hours, by which time the precipitate had settled. The unsaponifiable matter gave a slight precipitate when treated in the same way; 0.5 g. of crystalline chlorophyll gave no precipitate. Accordingly the necessary correction has been made when calculating the ratio of glycerol to fatty acids.

*Estimation of glycerol.* The acetone-ether-soluble material of batch *G* from autumn sown cabbage contained 39.4 % of fatty acids and 28.7 % of unsaponifiable matter; that from the spring sown cabbage of batch *M* contained 26.8 % of fatty acids and 41.1 % of unsaponifiable matter.

0.6030 g. of acetone-ether-soluble material from batch <i>G</i> :	0.1380 g. AgI = 0.0540 g. glycerol
0.6600 g. "	" <i>M</i> : 0.1148 g. AgI = 0.0449 g. "
0.3080 g. of unsaponifiable matter <i>G</i> :	" 0.0150 g. AgI = 0.0059 g. "
0.8268 g. " " <i>M</i> :	" 0.0446 g. AgI = 0.0175 g. "
Found: $\frac{\text{fatty acids}}{\text{glycerol}}$	$\frac{4.7}{1} \quad \frac{4.5}{1}$

The mono-, di- and tri-glycerides of the  $\text{C}_{18}$  saturated acids give the ratios 3.1/1, 6.2/1 and 9.3/1. A discussion of the glycerides present in the original ether extract will be held over until the analysis of the fraction which may contain hydroxy-acids is complete.

## SUMMARY.

The fatty acids of the cytoplasm of the cabbage leaf have been studied; the greater part consists of the unsaturated acids linolenic and linolic acids; palmitic and stearic acids constitute the saturated acids; the presence of oleic acid has not been proved. The ratio of fatty acids to glycerol is 4.6 : 1.

Part of the expenses of the research described in this and the two preceding papers of the series was defrayed by a grant from the Royal Society. One of us (A. C. C.) is indebted to the Department of Scientific and Industrial Research for a full time grant.

## REFERENCES.

- Chibnall and Channon (1927, 1). *Biochem. J.* **21**, 225.  
—— — (1927, 2). *Biochem. J.* **21**, 233.  
Levene and Rolf (1922). *J. Biol. Chem.* **51**, 507.  
Lowkowitch (1921). *Chemical technology of oils, fats and waxes*, p. 118.  
Willstätter and Stoll (1913). *Untersuchungen über Chlorophyll* (Berlin).  
Zeisel and Fanto (1903). *Z. anal. Chem.* **43**, 549.

# LXX. OBSERVATIONS ON THE BIOCHEMISTRY OF "MUTTON BIRD" OIL<sup>1</sup>.

BY CHARLIE LYONS CARTER AND JOHN MALCOLM.

*From the Departments of Chemistry and Physiology, University of Otago, Dunedin, N.Z.*

*(Received April 1st, 1927.)*

MANY seabirds, especially those of the petrel family, express their annoyance at the approach of an intruder by ejecting the contents of their stomach. This material is usually of an oily nature and in several different parts of the world advantage has been taken of this to obtain an oil that can be put to various uses. Thus the inhabitants of St Kilda collect the oil of the fulmar petrel and on the coasts of Tasmania and New Zealand a "stomach" oil is obtained from birds of the same family.

"Mutton bird" oil, the subject of this paper, is a mobile, reddish brown oil obtained in New Zealand from the petrel, *Aestrelata lessoni*, which nests on certain small islands off the south coast. When about to leave the nest, the young birds are killed for food by the Maoris, and if suspended head downwards a small quantity of the oil mixed with watery fluid can be collected as it flows from the beak. The oil can also be obtained in the same way from the parent birds, and the opinion is widely held that it is a nutrient fluid secreted by the parent birds and fed to the young. It occurred to us that another explanation might be that the oil represents an indigestible residue of the fatty fish (sprats, shell-fish, etc.), on which the petrel is said to feed during the nesting period.

To test this we were led to study the digestibility of the oil and from that we proceeded to trace as far as we could the fate of the oil during absorption and its behaviour when introduced parenterally. As yet we have not succeeded in unravelling the problems that presented themselves but we think it advisable to publish what we have done.

One of us (C. L. C.) has already published [1921] some work on the chemistry of the oil, and has shown that it has a close resemblance to sperm oil and consists largely of cetyl oleate together with esters of related alcohols and acids. It contains only traces of glycerides and a small amount of cholesterol. When our work began, we were not aware of any paper on the fate of cetyl esters in the body beyond the well-known work of Munk [1891],

<sup>1</sup> This paper was despatched from New Zealand before the receipt in that country of Rosenheim and Webster's paper on the stomach oil of the fulmar petrel [*Biochem. J.* (1927), **21**, 111].—EDITOR.

who showed that spermaceti (cetyl palmitate) is absorbed and appears in the thoracic duct as palmitin. According to Hutchison [1922] spermaceti was at one time used medicinally as an aid to fattening and was said to be well borne and not difficult to absorb. Purdy [1918] reports that in Tasmania an emulsion of mutton bird oil and almond oil had gained at one time a considerable reputation in the treatment of phthisis.

Thomas and Flaschenberger [1923], in work on the absorption of cetyl alcohol and cetyl acetate in dogs, found that a dog could absorb 4 to 6 g. of cetyl alcohol per day on a daily intake of 20 g. and about the same amount of the ester. They call attention to the difficulty in understanding how the cetyl alcohol, a substance insoluble in water and having such a high melting point ( $50^{\circ}$ ), can be absorbed, and they suggest that it may be due to combination with the desoxycholic acid of the bile, which Wieland and Sorge [1916] have shown can unite chemically with various similar substances to form compounds that are water-soluble.

A recent paper by Channon [1926] on the absorption and fate of the unsaturated hydrocarbon "squalene" (the "spinacene" of Chapman) is also of interest in this connection, for with both squalene and cetyl alcohol there is the same difficulty of understanding how absorption can occur.

#### DIGESTION *IN VITRO*.

The oil being of the nature of a fluid wax is not easily emulsified. The best results were obtained with gum acacia as recommended by Lueders and Bergeim [1923]. Toluene was added to inhibit bacterial action. The lipase preparation used was a fresh pig's pancreas, minced and pounded in a mortar with a small quantity of bile, then strained through cheese cloth. When 2 g. of this was added to 10 cc. of mutton bird oil emulsion made slightly alkaline to litmus, the mixture became distinctly acid in 17 hours, and on being made alkaline again developed an acid reaction. After 42 hours, it was made alkaline and extracted with ether. On comparing the weight of this extract with that of the fatty acids obtained from the alkaline residue it was found that 42 % of the original oil had been digested, while a control with olive oil emulsion and the same amount of lipase showed 83 % digestion. In another experiment, at the suggestion of Professor Inglis, we added lithium carbonate to the prepared tubes so as to provide a continuous supply of base to neutralise the fatty acids. In 3 days there were formed 3.416 g. fatty acid from 11.0 g. mutton bird oil, while a control with boiled ferment yielded 0.1 g. fatty acid from 10.5 g. of the same oil. This represents 50 % digested.

We could quote several other experiments but these are enough to show that the mammalian pancreatic lipase can hydrolyse the cetyl esters found in this oil, although the action appears to be slow as compared with that on glycerides. We have not had the opportunity to test the lipase of the bird itself under satisfactory conditions.



## ABSORPTION.

*Ducks.* Unsuccessful attempts, lasting over a week, were made to induce two ducks to feed on a paste made of pollard containing about 5 % of mutton bird oil.

*Cats.* A uniform diet of minced meat, porridge and milk was used and the oil was administered as an emulsion by stomach tube under light anaesthesia. The faeces were collected in 2 to 3 day periods, and extracted with ether after being finely broken up in water. The ether-soluble material was then saponified and the unsaponifiable fraction extracted with ether. In the second experiment the residue of faeces after the first ether extraction was saponified and again extracted so that we could be tolerably certain of having obtained all the cetyl alcohol excreted. The results are shown in Table I.

Table I. *Faeces (cat).*

	No.	Weight g.	Total ether soluble g.	Total unsaponi- fiable g.	Remarks
<i>First experiment:</i>					
Preliminary, 4 days	I	38	—	1.55	—
Experimental, 2 "	II	30	7.345	5.435	About 22 g. M.B. oil given
Subsequent, 4 "	III	?	1.945	1.415	—
<i>Second experiment:</i>					
Preliminary, 2 days	I a	23	2.69	1.58	—
Experimental, 2 "	II a	51	8.11	6.14	About 19 g. M.B. oil given
Subsequent, 3 "	III a	39	1.21	0.825	—

It will be noticed that in each experiment there was a large increase in the output of unsaponifiable matter, but the increase is less than the total unsaponifiable fraction (cetyl and other alcohols) of the oil given.

Thus in faeces II, if we subtract from the total unsaponifiable part (5.435 g.) the amount (= 0.77 g.) of such material that would normally be excreted on the diet given during the same time, there remain 4.7 g., whereas the unsaponifiable alcohols in the 22 g. of oil administered amount to 8.4 g.

Similarly, in the more satisfactory second experiment, II a, the gross output is 6.14 g. unsaponifiable matter, from which we may subtract 0.8 g. as the normal output, leaving only 5.34 g. as compared to 7.22 g. of unsaponifiable alcohols in the 19 g. of oil given.

In addition, we were convinced at the time that much of the unsaponifiable matter of the experimental periods consisted of cholesterol but unfortunately we were unable, owing to other work, to differentiate between the cholesterol and the alcohols of the mutton bird oil.

Although these two experiments were faulty because of the unnecessarily large doses given, the anaesthesia, and the incomplete analysis, they showed that the oil had no serious effect on the health of the cat and that a certain amount of the cetyl and other alcohols had been absorbed.

*Rats.* I. Three young rats, kept on a fairly uniform mixed diet, received daily doses of approximately 0.08 g. mutton bird oil administered by a pipette as in vitamin experiments. Three others of the same litter, similarly fed, were given the same amount of olive oil. During 16 days they received in all 3.12 g. of the oils. A sample of 25 g. of faeces from each group was boiled with acid alcohol to break up possible soaps; the alcohol was driven off and the residue extracted with ether. The resulting ether-soluble substances weighed in the mutton bird oil group 2.49 g., in the olive oil group 2.89 g. This material was then saponified and extracted with ether—the unsaponifiable fraction weighed 0.45 g. in the former, and 0.43 g. in the latter, and consisted only of sterols.

II. In this second experiment a similar procedure was adopted but the dose was increased so that in 20 days the same three rats received 11.47 g. mutton bird oil, which should have yielded 4.36 g. cetyl and other alcohols. The total faeces were collected and the unsaponifiable portion carefully examined. It weighed 1.523 g. and the first, second, and third crystallisations yielded crystals melting at 137° and in all probability consisted of cholesterol. No evidence of cetyl alcohol was obtained. The fatty acids of the faeces gave an iodine number of 77.8 as compared to 178 for mutton bird oil acids.

III. From the foregoing results we concluded that a rat is able to dispose of at least 0.2 g. mutton bird oil per day, and a further experiment was made in which the dose was increased to 0.5 g. per day. To three adult rats, a total of 12.33 g. of the oil was given over a period of 8 days. The unsaponifiable matter in this amount of oil would be about 4.7 g., while the unsaponifiable matter in the faeces collected during the 8 days weighed only 0.66 g. and fractional crystallisation and melting point determinations again showed that the bulk of it consisted of sterols. A trace of cetyl alcohol was found, enough to be identified by its melting point but not enough to weigh satisfactorily. The iodine value of the fatty acids of the faeces was 67.4 (Wijs).

In these experiments on rats the practical absence of cetyl alcohol and the low iodine value of the fatty acids indicate a very complete absorption of both components of the mutton bird oil up to approximately 3.3 g. per kg. body weight.

*Cetyl alcohol experiment.* It occurred to us that it would be interesting to test the absorption of cetyl alcohol by itself under similar conditions. A weighed amount of pure cetyl alcohol was mixed with olive oil to the extent of 15.74 %. When slightly warmed this mixture was sufficiently fluid to be administered by pipette as in the foregoing experiments. Two of the same rats as were used for Exp. III were given daily doses of the mixture over a period of nearly 3 weeks, during which they received in all 2.36 g. cetyl alcohol. The total faeces, collected and treated in the same way as for Exp. III, yielded 0.46 g. unsaponifiable matter from which 0.192 g. cetyl alcohol was recovered by crystallisation and recognised by its melting point, so that 91 % was absorbed, or at least disappeared. As compared to Exp. III, however, the absorption

of cetyl alcohol is not so good as that of the mixed alcohols of mutton bird oil, for in Exp. III the alcohols present in the daily dose of oil amounted to 0.19 g. (38 % of 0.5 g.) and this was almost completely absorbed—while, in the cetyl alcohol experiment, the daily dose was only one-third as much (0.062 g. per rat per day) and yet about 9 % remained unabsorbed.

#### FATE OF THE MUTTON BIRD OIL DURING AND AFTER ABSORPTION.

Having satisfied ourselves that the oil can be digested and absorbed, we next proceeded to investigate in what tissues or organs the simple chemical change of cetyl or other higher alcohol into fatty acid or other substance occurs. That it takes place naturally in the petrel cannot be doubted for these alcohols are absent from the body-fat of the bird.

To avoid repetition it may be stated here that the method used in estimating the alcohols was to saponify carefully and completely. The unsaponifiable fraction was then separated from the soaps by shaking with ether in a separating funnel and the constituents estimated by fractional crystallisation, weighing and determination of melting points. A certain amount of loss no doubt occurred, for from a mutton bird oil emulsion of known strength in one test case we recovered only 85 % of the alcohols.

(a) *Intestinal epithelium.* From the investigations of Munk on fat absorption it has long been known that cetyl alcohol does not appear in the chyle of the thoracic duct while spermaceti is being absorbed, and the intestinal epithelium has commonly been credited with the ability to transform this and other unusual fats into forms natural to the animal. In our experiments the small intestine of the rabbit with about half an inch of attached mesentery was excised, and, after washing out with Locke's fluid, filled with an emulsion of mutton bird oil of known strength to which had been added a strained suspension of the animal's own mashed pancreas and gall bladder bile. The caecal end of the intestine was then joined to the duodenal end by a very short piece of glass tubing, and the whole was immersed in a bath of warm oxygenated Locke's fluid. For from 4 to 6 hours the intestine continued to show movements, and no doubt a certain amount of circulation of the intestinal contents took place. A control of the same amount of emulsion plus pancreas and bile was kept in a beaker in the same bath.

On examining the intestinal contents and the control we recovered the cetyl alcohol to about the same extent in each, and in one case the ratio of the alcohol to the fatty acid was also examined and found to be unchanged. Four such experiments were done and in no case did we find any evidence that the surviving intestinal epithelium had affected the cetyl alcohol. It may be noted, however, that the amount of digestion may have been small, the leucocytes in the wall would be diminished, and the pumping action of the villi impaired owing to there being no pressure of blood in the blood vessels, so that the experiments did not exactly imitate the natural conditions

of absorption. The method used here was based on that used by Cohnheim [1911] in his experiments on intestinal absorption in fishes.

(b) *Parenteral administration.* This was tried both subcutaneously and intraperitoneally. A pregnant cat received 16 g. pure mutton bird oil under the skin of the back in two doses, one 14 days after the other, and both as nearly as possible in the same area. Seven days after the second dose the animal was killed.

At the site of the injection the effect was easily recognised by the presence of a deposit of canary-yellow fatty matter. The subcutaneous tissues were matted together so that the skin did not separate easily. The yellow colour could be traced for a considerable distance headwards and ventrally so that it infiltrated the mammary gland on the side of the injection. Wherever it had penetrated it seemed to have caused increased vascularity, probably inflammatory. The uterus contained five embryo kittens, and in each case there were streaks of the same yellow colour in the foetal membranes. Three samples of fat and subcutaneous tissue were taken for analysis, (I) from the actual site of injection, (II) from coloured fatty tissue near the mammary gland, and (III) normal fat from the opposite side of the body. The results of examining these are given in Table II.

Table II.

	Ether-soluble substance g.	Unsaponifiable matter g.	Fatty acids g.	Iodine value of fatty acids
I	13.234	1.708	10.222	91.4
II	14.560	1.438	8.326	90.5
III	12.280	Traces only	Not taken	

The unsaponifiable matter found in these samples consisted of practically pure cetyl alcohol and the figures indicate that the injected oil that was recovered had undergone little or no change. It had merely become mixed with the fatty tissue. Thus in samples (I) and (II), if a calculation is made of the quantity of fatty acid corresponding to the unsaponified alcohols found (in ratio of 60 : 38, which is the ratio in mutton bird oil), and if the iodine value of the fatty acids of the oil (178) and of the cat's normal fatty acids (63) be applied to these respective amounts, the iodine value of the mixture should be 93 to 94, which corresponds fairly well with the figures found, viz. 90.5 and 91.4. We did not attempt to recover all the oil injected because it had spread so widely. The general distribution of the streaks of yellow oil indicated transportation along the lymphatics. We have no explanation to offer of the well-marked presence of the same material in the foetal membranes.

For intraperitoneal injection we first used a cat. Nine g. of neutralised and sterilised oil were injected, and the animal killed 3 days later. The surfaces of the abdominal organs as well as the omentum and mesentery were found covered irregularly with a greasy, yellow, fibrinous deposit. As far as possible

this was washed out with alcohol followed by ether without extracting the deeper layer of colourless fat such as that surrounding the kidneys. The unsaponified alcohols (cetyl, etc.) recovered corresponded to that present in only 3.7 g. of the oil as against 9.0 g. injected. The iodine value of the fatty acids of the ethereal and alcoholic washings showed a rise as compared to normal.

On opening the thorax two small lymph glands were found in the anterior mediastinum. They were stained yellow like the abdominal organs and this also indicates lymphatic transportation.

In a later experiment we used a litter of young rats, and injected into the peritoneal cavity of each 2 cc. mutton bird oil representing 0.67 g. cetyl and other alcohols. One as a control was killed within a few minutes, and the whole carcase was minced and examined for the alcohols in the usual way (saponification, extraction with ether, etc.). The amount recovered from the control was only 0.35 g. In the two others similarly injected and examined at the end of 4 weeks and 5 weeks we recovered only 0.13 g. and 0.14 g. respectively. This probably indicates that some part of the unsaponifiable material (oleyl alcohol?) underwent metabolic change more readily than the cetyl alcohol which accordingly persisted longer in the body of the rat.

The fate of parenterally introduced fat in general was too large a subject for us to investigate, but our results agree with those of Binet and Verni [1925], for, before we had read the abstract of their paper, we had come to the conclusion that the best explanation of our experimental results was that the oil had excited irritative and mildly inflammatory changes which had led to ingestion of the oil by leucocytes and so to its removal from the site of injection; further, that the oil then underwent changes which led to loss of the characteristics of both parts of the ester. The first (subcutaneous) experiment shows that the esters are removed or changed without dissociation of their components—unlike what may occur in the case of cholesteryl oleate in the alimentary tract [Faust and Tallquist; quoted by Leathes, 1910].

*Influence of the liver.* In the hope of finding some fermentative action on the part of the liver which might change the alcohols, we made one experiment on freshly killed liver incubated with an emulsion of cetyl alcohol. The alcohol was almost quantitatively recovered from this and from the control of boiled liver.

#### VITAMIN A CONTENT.

The widely held opinion that the "stomach oil" plays a special part in the nourishment of the young bird led one of us (J. M.) to test its vitamin A activity, and some of the results have been already published [1926].

In the first set of experiments it was found that 0.04 g. and in some cases 0.02 g. daily was sufficient to produce rapid growth and to cure eye trouble in rats on a basal diet lacking in vitamin A. Later experiments on other samples have not always given the same result. Thus a quantity of oil pro-

cured in May 1925 gave maintenance only in doses of 0.06 g. A sample obtained in December 1925 gave good growth when administered at the same dosage. A sample examined in 1926 gave little or no growth. Such variations in activity are of course common in other cases and may have been due to diet, but we have reason to believe that one of the samples may have been subjected to some "refining" treatment by the Maoris before being delivered to us.

An investigation of the alcohols of mutton bird oil on lines suggested by the work of Drummond and Watson [1922], Fearon [1925], and Drummond, Channon and Coward [1925] has been commenced by one of us (C. L. C.) and his results will be published separately. It was found in general that those samples of oil which gave good results on growth also gave the sulphuric acid (purple) test, the trichloroacetic acid test with the oil in acetic anhydride, and the phosphorus pentoxide test.

On distillation of the unsaponifiable fraction of the oil at low pressure (4 mm.) and a temperature of 180°–200° a fraction consisting of practically pure oleyl alcohol came over, which gave the above colour reactions and which also caused a fair amount of growth in three rats which were being fed on basal diet containing oxidised cod-liver oil (20 hours) as source of vitamin D. These preliminary experiments showed that something came over with the oleyl alcohol, which promotes growth, though in two of the rats the eye symptoms persisted. Thus in a doe rat (*Ag* II, 3) 0.2 g. distillate (oleyl alcohol) daily for 4 days and after an interval of a week, during which the weight began to rise, the same dose repeated daily for 5 days caused ultimately a rise to 115 g. from 92 g., the weight when treatment began.

In another doe (*Ah* IV, 1) treated similarly in the same cage the weight rose from 66 g. to 87 g. and in a third (*Ad* V, 2) from 90 g. to 104 g. with cure of eye symptoms. In the two rats first mentioned the eye symptoms remained stationary.

#### ORIGIN OF THE OIL.

As stated above (p. 484) we at first thought it possible that the oil might be an indigestible residue of the bird's food. During the nesting period the petrels are believed by some to feed chiefly on the shoals of sprats and other members of the herring family that occur in these waters, by others they are believed to feed chiefly on shell-fish. In either case a residue of fatty material might result from gastric digestion of the food, and it has been pointed out by others that birds in general have a poor supply of lipases in their digestive secretions. Against this view, however, there is the evidence of a great degree of uniformity in the composition of the oil, and the low concentration of cholesterol.

During the course of investigations on the food value of New Zealand fishes we took the opportunity to examine the unsaponifiable part of many of the fish fats, liver oils, and shell-fish fats, on the chance of discovering a

clue to the source of the cetyl alcohol—but without success. The chief unsaponifiable alcohol in these oils is cholesterol.

Another view widely held is that the oil is secreted by the parent birds for the nourishment of the young. Such secretion is, we think, very unlikely. The petrel has no crop such as the pigeon has, and the oil is not accompanied by the protein that one would expect if this were a nutritive fluid like pigeon's milk.

As the research proceeded, a third view has gradually impressed itself on us as more likely than either of these, and that is, that the oil is the excess of tail gland secretion swallowed by the parent bird and more or less accidentally included in the material fed to the young. Possibly, also, the young bird may produce and swallow some of its own tail gland secretion. In support of this we may call attention to the fact that cetyl and octadecyl alcohols have been found to occur up to 40 % in the "fat" extracted from the tail gland of geese [Metzner, 1907]. Being unable at present to obtain adult mutton birds, we have investigated the tail gland of ducks. In these we find it easy by mechanical stimulation to cause oily drops to appear on the feathered apex of the nipple-like projection which forms the outlet for the gland. We procured a number of duck tail glands from a local poultry dealer and tested the ethereal extract of these for vitamin A. Doses of 0.1 g. gave no evidence of its presence, but this result is not conclusive, for all would depend on the food of the ducks. That skin secretions may contain vitamin A has already been shown by Gudjonsson [1926]. During the coming winter we hope to secure evidence on this point from the mutton bird itself.

#### SUMMARY AND CONCLUSIONS.

The oil obtained from the stomach of the mutton bird is capable of being digested, absorbed and utilised by mammals. It may contain vitamin A in high concentration but the amount present is variable. The cetyl, oleyl and other alcohols of the oil are changed during or after absorption so that they can no longer be detected, but experiments on the action of surviving intestinal wall and liver cells failed to show where the change occurs.

On parenteral injection, subcutaneously and intraperitoneally, there is evidence that no local dissociation of the esters occurs, and the oil appears to be taken up by leucocytes and to travel by the lymphatics.

The view is put forward that the stomach oil of the chick is the tail gland secretion more or less accidentally swallowed.

The expenses of this research were defrayed to a large extent by a grant from the New Zealand Institute, to whom our thanks are due.

## REFERENCES.

- Binet et Verni (1925). *Compt. Rend. Soc. Biol.* 83. [*Physiol. Abstr.* 10, 473.]  
Carter (1921). *J. Soc. Chem. Ind.* 40, 220 T.  
Channon (1926). *Biochem. J.* 20, 400.  
Cohnheim (1911). *Z. physiol. Chem.* 76, 293.  
Drummond, Channon and Coward (1925). *Biochem. J.* 19, 1047.  
Drummond and Watson (1922). *Analyst*, 47, 314.  
Fearon (1925). *Biochem. J.* 19, 888.  
Gudjonsson (1926). *Amer. J. Physiol.* 75, 533.  
Hutchison (1922). Food and dietetics, 564.  
Leather (1910). The fats, 120 (London).  
Lueders and Bergeim (1923). *Amer. J. Physiol.* 66, 297.  
Malcolm (1926). *Trans. New Zealand Inst.* 58, 650.  
Metzner (1907). *Nagel's Handb. Physiol.* 2, 388.  
Munk (1891). *Virchow's Arch.* 123, 230 and 486.  
Purdy (1918). *Brit. Med. J.* i, 174.  
Thomas and Flaschenberger (1923). *Skand. Arch. Physiol.* 43, 1.  
Wieland and Sorge (1916). *Z. physiol. Chem.* 97, 1.



# LXXI. THE INFLUENCE OF THE FATTY ACIDS AND HYDROXY-ACIDS AND THEIR SALTS ON ALCOHOLIC FERMENTATION BY LIVING YEAST.

## PART II. PROPIONIC, BUTYRIC, ISO-BUTYRIC, GLYCOLLIC, LACTIC, HYDROXY-ISO-BUTYRIC, $\alpha$ - AND $\beta$ -HYDROXYBUTYRIC ACIDS AND THEIR SODIUM SALTS.

By HIDEO KATAGIRI.

*From the Biochemical Department, Lister Institute, London.*

*(Received April 4th, 1927.)*

In his previous paper [1926], the author investigated the rate of fermentation of sugar by living yeast with buffer solutions composed of acetic acid or formic acid and their Na, K and  $\text{NH}_4$  salts. These lowest homologues of the fatty acids exert a specific action upon the rate of fermentation and it is suggested that a hyperbolic relation exists between the concentrations of these acids in the media and the rates of fermentation. Their inhibiting effect is not the same, formic acid being 5-8 times as potent as acetic acid in diminishing the rate of fermentation.

As to the inhibiting action of fatty acids upon the rate of fermentation, Bial [1902] considered that this was due to the hydrogen ion; consequently their degree of dissociation would decide the requisite amount of the fatty acids to stop the fermentation. This conclusion was confirmed by Hägglund [1914] with acetic and butyric acids. On the other hand, Johannessohn [1912] pointed out that the inhibiting effect of the fatty acids appeared to be due to the undissociated molecule rather than to the dissociation products. With an acetic acid and Na acetate buffer solution, Euler and Heintze [1919] confirmed Johannessohn's observations. On the other hand, according to Duggan [1885], the degree of disinfecting action of fatty acids upon *B. subtilis* is inversely proportional to their molecular weights.

In the present paper, experiments were at first instituted with buffer solutions composed of higher homologues of fatty acids and their salts, in order to ascertain what influence would be exerted on the rate of fermentation, whether a hyperbolic relation would exist between the concentration of the acids and the rate of fermentation, and which acid would be more potent, the lower homologue or the higher one.

## METHOD AND RESULTS.

Full details of the experimental technique were given in the previous paper [Katagiri, 1926]. The principal points are as follows: fermentation was carried out by 2 g. of pressed top yeast with 30 cc. of the various buffer solutions containing 1.5 g. of glucose after saturation with  $\text{CO}_2$  and keeping the temperature at  $25^\circ$  in a water-bath. The amount of evolved  $\text{CO}_2$  was observed at intervals of 5 minutes. For the rate of fermentation, the average number of cc. of  $\text{CO}_2$  evolved in 5 minutes during about 1 hour's fermentation was chosen and its value was corrected for variation in the fermenting power of the yeast by a standard fermentation which was always carried out in presence of a 0.2 *M* acetate solution at  $p_{\text{H}}$  4.7. Each  $p_{\text{H}}$  value was determined by the capillator method both at the beginning and at the end of the fermentation, after saturation with  $\text{CO}_2$ .

The amount of free acid was calculated according to the amount added plus the amount that was produced from the salt in the process of saturation with  $\text{CO}_2$ . The latter amount of the acid was calculated from the amount of  $\text{CO}_2$  evolved by the addition of HCl of suitable strength to a mixture having the same composition as the solution used in the fermentation.

The results obtained with propionic acid, *n*-butyric acid and *iso*-butyric acid are contained in Table I and Fig. 1.

Table I.

Acid	Total concentration Propionate ( <i>M</i> )	Ratio (salt/acid)			
		1 : 4	1 : 1	3 : 1	1 : 0
A. Propionic	0.25	0.200	0.125	0.062	0.015
	0.2	0.160	0.100	0.050	0.013
	0.1	0.080	0.050	0.025	0.010
	0.05	0.040	0.025	0.012	0.005
	0.025	0.020	0.012	0.006	0.003
	Butyrate ( <i>M</i> )	Ratio (salt/acid)			
		1 : 3	1 : 1	3 : 1	1 : 0
B. Butyric	0.5	0.375	0.250	0.126	0.018
	0.2	0.150	0.100	0.050	0.010
	0.1	0.075	0.050	0.025	0.006
	0.05	0.037	0.025	0.012	0.004
	0.025	0.019	0.012	0.006	0.002
	<i>iso</i> -Butyrate ( <i>M</i> )	Ratio (salt/acid)			
		9 : 11	1 : 1	3 : 1	1 : 0
C. <i>iso</i> -Butyric	0.5	0.275	0.250	0.125	0.019
	0.2	0.110	0.100	0.050	0.011
	0.1	0.055	0.050	0.025	0.006
	0.05	0.027	0.025	0.012	0.005
	0.025	0.013	0.012	0.006	0.001

The curves (Fig. 1) obtained when the rate of fermentation with the buffer solutions composed of propionic acid, *n*-butyric acid and *iso*-butyric acid with their sodium salts is plotted against the  $p_{\text{H}}$ , are of much the same character as was observed with formate and acetate buffer solutions in the previous paper. The  $p_{\text{H}}$  values did not change by more than 0.3  $p_{\text{H}}$  during the fermentation and the mean values have been taken.

The concentration of the buffer solutions again appears to have a remarkable effect on the rates of fermentation, when these are compared at constant  $p_H$ , the rate of fermentation increasing rapidly as the concentration of the buffers diminishes. As in the case of acetate-acetic acid and formate-formic acid buffer solutions, however, the rate of fermentation is controlled by the amount of free acid and is almost independent of the total concentration. This is well brought out in Fig. 2. A hyperbolic relation can again be supposed to exist between the rate of fermentation and the concentration of acids in the medium.

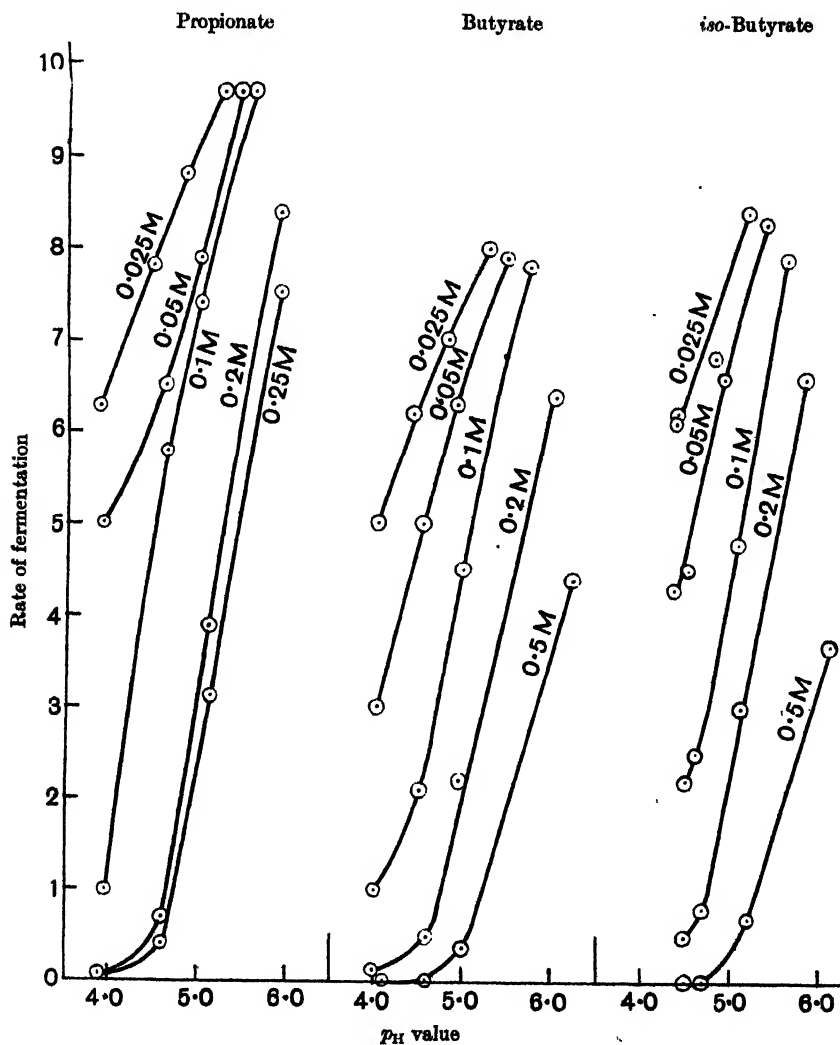


Fig. 1.

For a direct comparison of the inhibiting effects of the various acids, the amount of each acid may be compared in two ways: one in which the rates

of fermentation are diminished to such degrees as  $2/3$ ,  $1/2$ ,  $1/3$ ,  $1/4$  or  $1/10$  of the value observed with the least quantity of each acid, and the other in which constant rates of fermentation are observed, *e.g.* 1, 2, 5 and 8 cc.  $\text{CO}_2$  per 5 minutes. These values are not constant, but formic acid is relatively more effective at every concentration, while the other fatty acids have nearly the same effect. As examples, the concentrations required to diminish the rate of fermentation to  $1/4$  and to produce a rate of fermentation of 5 cc. per 5 minutes are given in Table II.

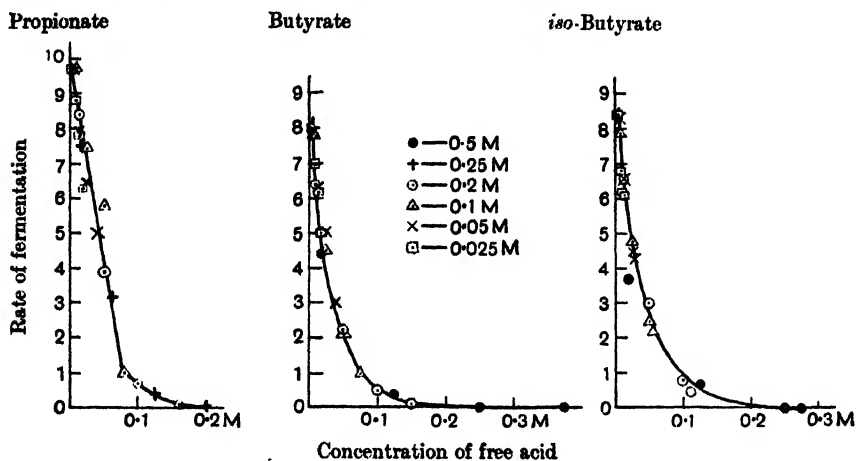


Fig. 2.

Table II.

Acid	Concentration of acid required to diminish the rate of fermentation to 1/4		Concentration of acid with which a rate of 5 cc. per 5 mins. was observed		Concentration of acid required completely to inhibit fermentation				Dissociation constant of the acids (25°)
					Bial [1902]		Johannessohn [1912]		
	<i>M</i>	Relative	<i>M</i>	Relative	<i>M</i>	Relative	<i>M</i>	Relative	
Formic	0.0100	1	0.007	1	0.016	1	0.0139	1	$21.4 \times 10^{-5}$
Acetic	0.0500	5.0	0.030	4.3	0.041	2.6	0.0841	6.0	$1.86 \times 10^{-5}$
Propionic	0.0675	6.7	0.040	5.7	0.066	4.1	0.1080	7.8	$1.4 \times 10^{-5}$
<i>n</i> -Butyric	0.0525	5.2	0.020	2.9	0.050	3.1	0.0926	6.6	$1.48 \times 10^{-5}$
<i>iso</i> -Butyric	0.0500	5.0	0.020	2.9	—	—	0.0926	6.6	$1.5 \times 10^{-5}$

These numerical relations of the fatty acids agree in general with the results obtained by Bial [1902], Hägglund [1914] and Johannessohn [1912], all of whom carried out the fermentation in absence of a buffer solution.

Vermast [1921] found that the disinfecting action of benzoic acid on *Bacillus coli communis* was controlled by the concentration of the undissociated acid and could be completely accounted for on the basis of the Meyer-Overton partition theory, employing the partition coefficient between water and benzene and basing the calculation on the concentration of undissociated acid.

However, the results obtained at room temperature by Kuriloff [1898] and Georgievics [1913] on the partition of the fatty acids between water and benzene, which are quoted below, show that there is no proportionality be-

tween their partition coefficients and the inhibiting action of the acids on fermentation, so that the relation found by Vermast for disinfection does not hold in the case of fermentation.

Acid	$C_1/C_2$	$C_1(1-\alpha)/C_2$	Author
Formic	460.5 - 340.8	455.7 - 339.5	Georgievics
Acetic	55.1 - 80.1	54 - 64	Kurloff
<i>n</i> -Butyric	0.81- 0.54	0.79- 0.54	Georgievics

where  $C_1$  and  $C_2$  represent the concentration of acid in water and in benzene, respectively,  $\alpha$  represents the degree of dissociation of the acid.

Thus, no reliable explanation has yet been put forward as to the effect of the fatty acids in diminishing the rate of fermentation, but it appears that the magnitude of the observed inhibiting effects of the various fatty acids is attributable, in a great measure, to their chemical nature.

#### HYDROXY-ACIDS.

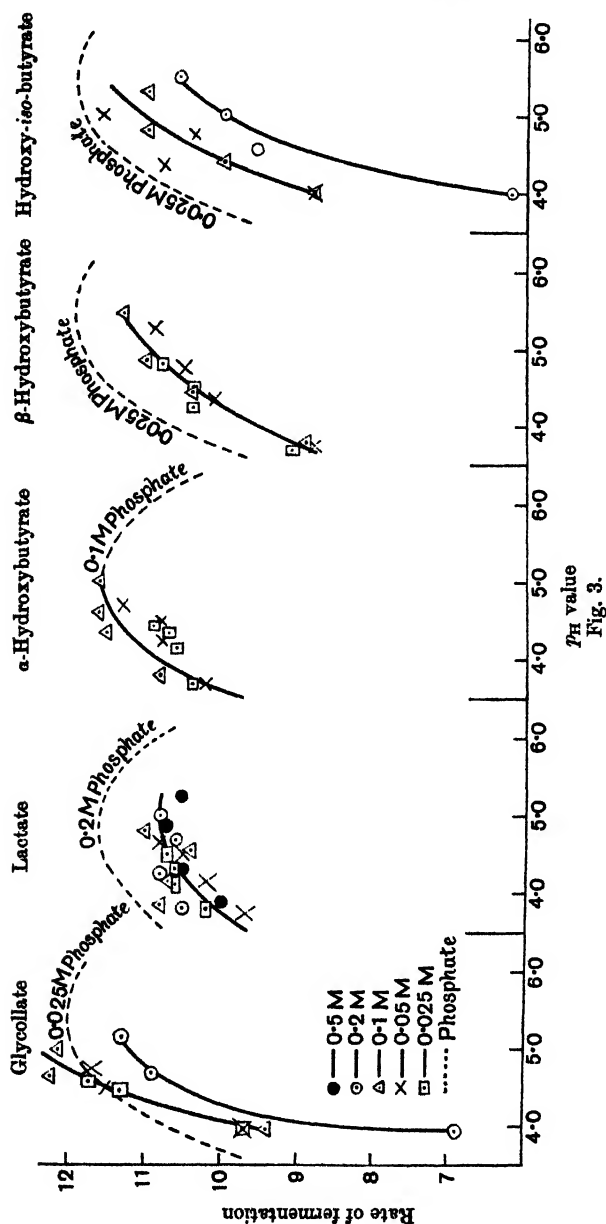
In order to ascertain the effect of the introduction of a hydroxy-group on the inhibiting properties of the fatty acids on fermentation, experiments were made in the same manner as with fatty acids with glycollic, lactic,  $\alpha$ -hydroxy-*n*-butyric,  $\beta$ -hydroxy-*n*-butyric and hydroxy-*iso*-butyric acids and their sodium salts.

The results are given in Fig. 3 and Table III, the mean values of  $p_H$  being taken as in Fig. 1.

Table III.

Acid	Total concentration Glycollate ( <i>M</i> )	Ratio (salt/acid)		
		5 : 1	15 : 1	1 : 0
A. Glycollic ( $K = 1.5 \times 10^{-4}$ at 25°)	0.2	0.033	0.012	0.004
	0.1	0.017	0.006	0.002
	0.05	0.008	0.003	0.002
	0.025	0.004	0.001	0.0004
	Lactate ( <i>M</i> )	Ratio (salt/acid)		
		2 : 1	5 : 1	15 : 1
B. Lactic ( $K = 1.38 \times 10^{-4}$ at 25°)	0.5	0.167	0.083	0.031
	0.2	0.067	0.033	0.012
	0.1	0.033	0.016	0.006
	0.05	0.016	0.008	0.003
	0.025	0.008	0.004	0.001
	$\alpha$ -Hydroxy- butyrate ( <i>M</i> )	Ratio (salt/acid)		
		3 : 1	18 : 3	39 : 1
C. $\alpha$ -Hydroxybutyric ( $K = ca. 0.45 \times 10^{-4}$ at 25°)	0.1	0.025	0.015	0.003
	0.05	0.012	0.007	0.001
	0.025	0.006	0.004	0.0005
				0.0003
	$\beta$ -Hydroxy- butyrate ( <i>M</i> )	Ratio (salt/acid)		
		1 : 2	2 : 1	5 : 1
D. $\beta$ -Hydroxybutyric ( $K = 0.39 \times 10^{-4}$ at 22°)	0.1	0.067	0.033	0.019
	0.05	0.033	0.016	0.011
	0.025	0.016	0.008	0.004
				0.002
	Hydroxy- <i>iso</i> -butyrate ( <i>M</i> )	Ratio (salt/acid)		
		2 : 1	5 : 1	15 : 1
E. Hydroxy- <i>iso</i> -butyric ( $K = 1.06 \times 10^{-4}$ at 25°)	0.2	0.067	0.033	0.012
	0.1	0.033	0.016	0.006
	0.05	0.016	0.008	0.003
				0.0007

When the rates of fermentation are plotted against the  $p_H$  (Fig. 3), the picture is totally different from that presented by the unsubstituted fatty acids (Fig. 1). Independently of the total concentration of the buffer solutions,



the values are found to be situated on regular curves which evidently approach a maximum and closely resemble those given by phosphates (see Fig. 4). The only exception is glycollic acid, which is intermediate in character between

the fatty and hydroxy-acids. These curves show that the rate of fermentation is very largely controlled by the hydrogen ion concentration, but that some other influence modifies this is clear from the fact that the curves are not identical and that the optimum  $p_H$  is not the same in each case. This points to some secondary effect due possibly to the specific nature of the acid. This is specially marked in the case of glycollic acid as will be seen from the curves in Fig. 3.

#### PHOSPHATE BUFFER SOLUTIONS.

In order to discuss whether the hydroxy-acids reveal any specific effect on the rate of fermentation, the author's interest was directed towards the inorganic buffer solutions containing phosphate or carbonate, which might serve as standards.

The results with Na phosphate buffer solutions are given in Table IV and Fig. 4. In this figure the  $p_H$  values measured at the end of the fermentation were used, since phosphate buffer solutions in the range of about  $p_H = 3-6$  are not strongly buffered unless the most concentrated solution (0.5 *M*) is used.

Table IV. *Sodium phosphate buffer solutions.*

	Total concentration of phosphate ( <i>M</i> )	Concentration of $H_2PO_4$ ( <i>M</i> )	Concentration of $NaH_2PO_4$ ( <i>M</i> )	Corrected rate of fermentation cc. per 5 mins.	$p_H$	
					Beginning	End
<i>Exp. 34</i>	0.5	0.050	0.450	6.8	2.6	2.6
	0.5	0	0.500	11.6	4.2	4.0
	0.5	0	0.490	11.5	4.9	4.6
	0.5	0	0.479	11.6	5.1	4.9
	0.5	0	0.389	9.3	6.1	6.1
	0.5	0	0.137	4.4	7.0	6.9
<i>Exp. 35</i>	0.2	0.020	0.180	7.0	2.7	2.7
	0.2	0.005	0.195	10.8	3.7	3.4
	0.2	0	0.200	11.1	4.6	3.8
	0.2	0	0.195	11.6	5.1	4.4
	0.2	0	0.163	10.7	6.1	6.1
	0.2	0	0.090	9.4	6.8	6.8
	0.2	0	0.089	7.6	6.9	6.9
<i>Exp. 36</i>	0.1	0.010	0.090	6.9	2.7	2.7
	0.1	0.002	0.098	10.3	3.7	3.5
	0.1	0	0.100	10.1	4.4	3.7
	0.1	0	0.097	11.1	5.1	4.1
	0.1	0	0.085	11.1	6.1	5.9
	0.1	0	0.060	10.1	6.5	6.5
	0.1	0	0.057	9.7	6.8	6.7
<i>Exp. 37</i>	0.025	0.0025	0.0225	7.4	2.8	2.9
	0.025	0.0006	0.0244	9.9	3.7	3.6
	0.025	0	0.025	9.7	4.2	3.7
	0.025	0	0.024	10.0	4.5	3.7
	0.025	0	0.022	11.4	5.7	4.4
	0.025	0	0.018	11.8	6.4	6.1
	0.025	0	0.019	11.8	6.5	6.0
	0.025	0	0.015	10.5	6.6	6.6
	0.025	0				

When the fermentation rates are plotted against the  $p_H$  (Fig. 4), it is seen that the curves for different concentrations of total phosphate are not identical; both the optimum  $p_H$  and the sharpness of the maximum vary. The 0.5  $M$  curve shows the sharpest maximum, whilst the least sharp is shown by the 0.2  $M$  curve.

In the present paper,  $p_H$  values are determined colorimetrically; consequently, when  $p_H$  values of various concentrations of a buffer solution are compared, a certain correction for the so-called salt error must be taken into

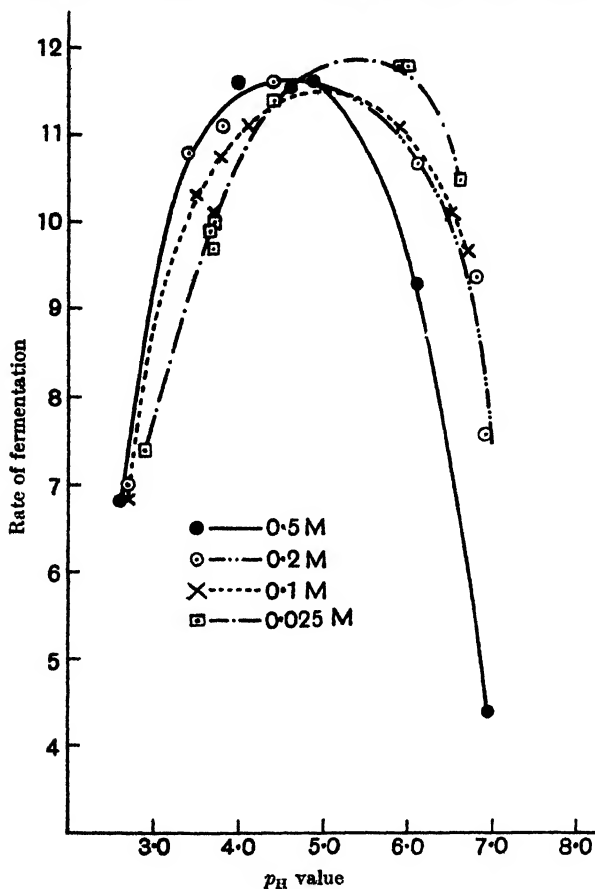


Fig. 4.

account. The correction for salt error becomes greater as the concentration differs from about 0.1  $M$  phosphate and the sign of the correction is different on each side of this concentration. In the case of 0.5  $M$  or 0.2  $M$  solution, the corrected value will be more acidic ( $p_H$  less) and in the case of 0.025  $M$  the corrected value will be more alkaline ( $p_H$  greater). [See Büllmann and Katagiri, 1927.]

From the present uncorrected colorimetric determinations which are tabulated below it is clearly seen that the optimum  $p_H$  moves to the alkaline



side as the concentration of the phosphate buffer solution diminishes. This change would be accentuated if the  $p_H$  values were corrected for "salt error" as just explained.

Concentration of phosphate buffer solution ( $M$ )	Optimum $p_H$
0.5	4.45
0.2	4.8
0.1	5.0
0.025	5.25

It has not yet been decided whether Michaelis' equations [1911] for amphoteric electrolytes are applicable in the case of living yeast to the rate of fermentation and the  $p_H$  value as applied by Michaelis and Davidsohn [1911] and Davidsohn [1913] to enzyme action, but it is interesting to note that in Table V, in which  $K$  is calculated from these equations for values of fermentation rate and  $p_H$  taken from the curve in Fig. 4 for 0.5  $M$  phosphate, the values of  $K$  obtained on each side of the maximum are approximately constant.

Table V. 0.5  $M$  phosphate buffer solutions.

$p_H$	Rate of fermen- tation cc. per 5 mins.	Relative rate of fermen- tation	$K_a$ for $\frac{[H^+]}{[H^+] + K_a} = \text{rel. rate}$	$K_b$ for $\frac{[OH^-]}{[OH^-] + K_b} = \text{rel. rate}$
*2.6	6.8	0.59	—	$0.28 \times 10^{-11}$
2.65	7.0	0.60	—	$0.30 \times 10^{-11}$
2.8	8.0	0.70	—	$0.27 \times 10^{-11}$
2.95	9.0	0.78	—	$0.25 \times 10^{-11}$
3.15	10.0	0.86	—	$0.24 \times 10^{-11}$
*4.0	11.6	1.00	—	—
Opt.				
4.45	11.6	1.00	—	—
*4.9	11.6	1.00	—	—
5.9	10.0	0.86	$0.21 \times 10^{-6}$	—
*6.1	9.3	0.80	$0.20 \times 10^{-6}$	—
6.35	8.0	0.70	$0.19 \times 10^{-6}$	—
6.5	7.0	0.60	$0.21 \times 10^{-6}$	—
6.65	6.0	0.52	$0.20 \times 10^{-6}$	—
6.85	5.0	0.43	$0.19 \times 10^{-6}$	—
*6.95	4.4	0.38	$0.20 \times 10^{-6}$	—

\* Represents the point of observation.

No simple relations have been found to exist between the rate of fermentation and the concentration of free  $H_3PO_4$ , or of  $NaH_2PO_4$ , or of  $Na_2HPO_4$ .

When the rates of fermentation with the hydroxy-acids are compared with those obtained with phosphate buffer solutions, a very close resemblance is observed to exist between them; *e.g.* between the curve with lactic acid and that of 0.2  $M$  phosphate; between those of  $\alpha$ -hydroxy-*n*-butyric acid and 0.1  $M$  phosphate; between those of  $\beta$ -hydroxy-*n*-butyric acid and 0.025  $M$  and between those of hydroxy-*iso*-butyric acid and 0.025  $M$  phosphate. From this resemblance it may be concluded that hydroxy-acids with the exception of glycollic acid do not exert any marked specific effect upon the rate of fermentation as compared with phosphate buffer solutions.

## SODIUM BICARBONATE.

Table VI shows the results obtained with various concentrations of sodium bicarbonate buffer solutions and with the corresponding concentrations of phosphate buffer solutions. In order to make the bicarbonate buffer solutions, NaOH solutions of the corresponding concentrations were used. For each concentration of  $\text{NaHCO}_3$ , only one  $p_{\text{H}}$  value is possible, since, under the conditions of the experiment, the solutions are saturated with  $\text{CO}_2$  at  $25^\circ$ . For the phosphate buffers  $\text{Na}_2\text{HPO}_4$  solutions were employed. Having added the requisite amount of sugar solution, they were saturated with  $\text{CO}_2$ , yeast was added and the fermentation was carried out.

Table VI.

*Sodium bicarbonate buffer solution.*

Exp. 38

Concentration of bicarbonate (M)	Corrected rate of fermentation cc. per 5 mins.	$p_{\text{H}}$ (corrected)	Relative rate of fermentation	$K_e$ for $\frac{[\text{H}^*]}{[\text{H}^*] + K_e} = \text{rel. rate}$
0.5	4.5	7.3	0.417	$0.70 \times 10^{-7}$
0.2	7.3	7.0	0.676	$0.48 \times 10^{-7}$
0.1	8.9	6.8	0.824	$0.34 \times 10^{-7}$
0.05	9.7	6.5	0.898	$0.36 \times 10^{-7}$
0.025	10.8	6.2	1.000	—

*Sodium phosphate buffer solution.*

Exp. 39

Concentration of phosphate (M)	Corrected rate of fermentation cc. per 5 mins.	$p_{\text{H}}$ (corrected)	Relative rate of fermentation	$K_e$ for $\frac{[\text{H}^*]}{[\text{H}^*] + K_e} = \text{rel. rate}$
0.5	4.4	6.7	0.373	$3.36 \times 10^{-7}$
0.2	7.6	6.6	0.644	$1.38 \times 10^{-7}$
0.1	9.7	6.5	0.822	$0.69 \times 10^{-7}$
0.05	10.8	6.3	0.915	$0.41 \times 10^{-7}$
0.025	11.8	6.1	1.000	—

The  $p_{\text{H}}$  values in Table VI were corrected for salt error and for the barometric pressure under which the saturation of  $\text{CO}_2$  was carried out from the values obtained electrometrically by Büllmann and Katagiri [1927] under the same experimental conditions.

In Fig. 5 (a) in which the rates of fermentation shown in Table VI are plotted against the  $p_{\text{H}}$ , it is clearly seen that the curve with bicarbonate buffer solutions is of a similar nature to that obtained with phosphate buffer solutions, but they are not perfectly identical, since in the latter curve the rate of fermentation diminishes more rapidly than in the former when the  $p_{\text{H}}$  value increases.

When the rates of fermentation are plotted against the concentration of total salts (Fig. 5 (b)), the bicarbonate and phosphate curves are again very similar, although they are not identical, but in the latter curve the rate of fermentation diminishes a little more rapidly as the concentration increases.

When Michaelis' formula for amphoteric electrolytes is applied to the

results obtained with bicarbonate and phosphate buffer solutions, the values of  $K_c$  (Table VI) vary as is to be expected in both cases with the concentration of total salt. When the values of  $K_c$  are plotted against the molar concentration of the salts (Fig. 6), a linear relation is found to exist. The inclination of the phosphate curve is greater than that of the bicarbonate curve as was to be expected from Fig. 5 (a) and (b).

Whilst the interpretation of these results is not very clear, it may be considered that with the bicarbonate buffer solution, the rate of fermentation is more exclusively controlled by the  $p_H$  value than with the phosphate buffer solution.

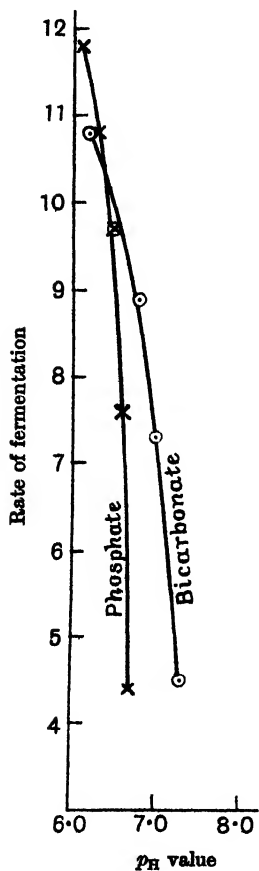


Fig. 5 (a)

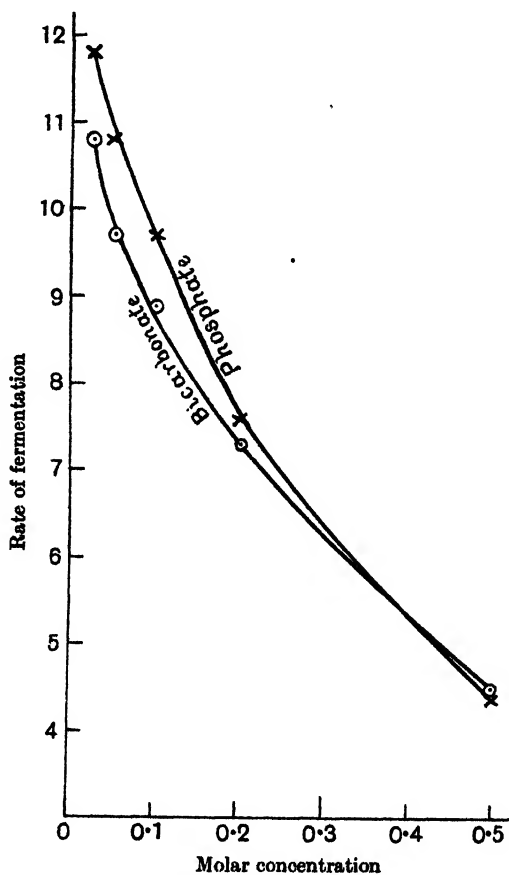


Fig. 5 (b)

#### SUMMARY.

(1) Fermentation by living yeast was observed with various concentrations of propionate, butyrate, *iso*-butyrate, glycollate, lactate,  $\alpha$ - and  $\beta$ -hydroxy-butyrate, hydroxy-*iso*-butyrate, phosphate and bicarbonate buffer solutions composed in each case of the acid and its sodium salt.

(2) The simple fatty acids have an inhibiting effect on the rate of fermentation, and it is suggested in each case that a hyperbolic relation exists between the rate of fermentation and the concentration of free acid.

(3) The specific effect of these fatty acids is nearly as great as that of acetic acid, but very much less than that of formic acid.

(4) The order of the specific effect of the fatty acids is not coincident with that of their partition coefficients between water and benzene or that of their molecular weights or dissociation constants. It is probably due, in a great measure, to the chemical nature of the acid itself.

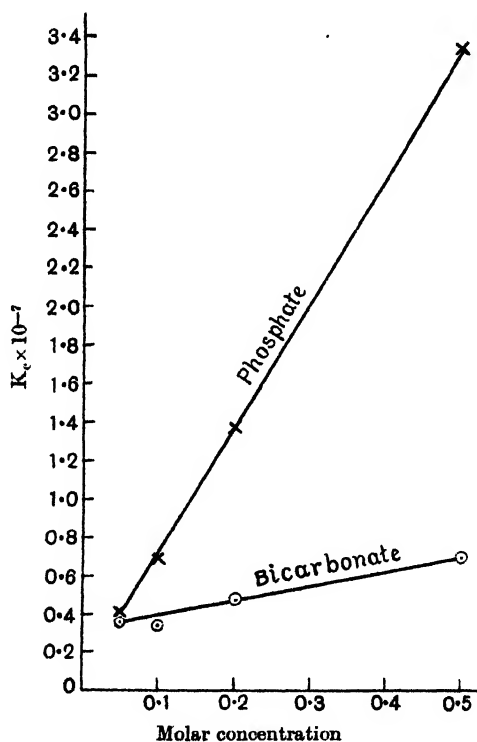


Fig. 6.

(5) No specific effect of the hydroxy-acids on the rate of fermentation as compared with phosphate buffer solutions was observed, except in the case of glycollic acid which had a slight inhibiting action similar to that of the simple fatty acids.

(6) Curves showing an optimum  $p_H$  value were obtained with phosphate buffer solutions of various concentrations. As in the case of enzyme action in general, the rates of fermentation at constant concentration of buffer solutions were observed to be principally controlled by the  $p_H$  value.

(7) Not only a different optimum  $p_H$  value but also a different sharpness of optimum  $p_H$  was observed with phosphate buffer solutions of different concentration. As the concentration diminished, the optimum  $p_H$  moved to the alkaline side.

(8) The effect of buffer solutions of sodium bicarbonate and carbonic acid is very similar to that of phosphate buffers, but the rate of fermentation is more exclusively controlled by the  $p_H$  value than is the case with the phosphate buffers.

I desire to acknowledge my great indebtedness to Prof. A. Harden for his continued interest and his valuable criticism throughout the whole of the investigation.

#### REFERENCES.

- Bial (1902). *Z. physikal. Chem.* **40**, 513.  
Billmann and Katagiri (1927). *Biochem. J.* **21**, 441.  
Davidsohn (1913). *Biochem. Z.* **49**, 247.  
Duggan (1885). *Amer. Chem. J.* **7**, 62.  
Euler and Heintze (1919). *Z. physiol. Chem.* **108**, 165.  
Georgievics (1913). *Z. physikal. Chem.* **84**, 353.  
Hägglund (1914). *Sammlung Ahrens-Herz (Stuttgart)*, **21**, 129.  
Johannessoehn (1912). *Biochem. Z.* **47**, 97.  
Katagiri (1926). *Biochem. J.* **20**, 427.  
Kuriloff (1898). *Z. physikal. Chem.* **25**, 419.  
Michaelis (1911). *Biochem. Z.* **33**, 182.  
Michaelis and Davidsohn (1911). *Biochem. Z.* **35**, 386.  
Vermast (1921). *Biochem. Z.* **125**, 106.

## LXXII. OXIDATIONS BY HYDROGEN PEROXIDE IN PRESENCE OF SULPHYDRYL COMPOUNDS.

By DOUGLAS CREESE HARRISON.

*From the Department of Pharmacology, Sheffield University.*

*(Received April 4th, 1927.)*

It has been shown recently by Thurlow [1925] and by Harrison and Thurlow [1926] that during certain biological oxidations such as that of hypoxanthine and succinic acid in presence of their respective oxidising enzymes, or sulphhydryl compounds in presence of traces of iron, the secondary or induced oxidation of substances such as nitrites, lactic acid and  $\beta$ -hydroxybutyric acid can be brought about. These secondary oxidations were shown to occur only in presence of a suitable peroxidase, *e.g.* milk peroxidase or ferrous iron. Thurlow [1925] gave evidence that these secondary oxidations were brought about by hydrogen peroxide formed during the oxidation of the primary system by the combination of active hydrogen with molecular oxygen. In the case of the primary oxidising system hypoxanthine with xanthine oxidase, hydrogen peroxide was actually detected in the solution. The hydrogen peroxide in presence of a suitable peroxidase or activator can then bring about the secondary oxidation of other substances. During the oxidation of the sulphhydryl compound, however, positive tests for the presence of hydrogen peroxide in the solution could not be obtained. This was explained by the fact that hydrogen peroxide can readily oxidise the sulphhydryl group and that, consequently, hydrogen peroxide formed during the oxidation of the sulphhydryl compound by oxygen would be capable of bringing about the oxidation of a further quantity of the sulphhydryl compound, and hence would rapidly be used up. Similarly, hydrogen peroxide formed in other primary oxidising systems such as hypoxanthine with xanthine oxidase can bring about the oxidation of a further quantity of the oxidisable substance hypoxanthine, though in this latter case the rate of disappearance of the hydrogen peroxide is slower, requiring the presence of the xanthine oxidase. It was shown, however, by Thurlow [1925] and Harrison and Thurlow [1926] that, in spite of the fact that part of the hydrogen peroxide formed during the primary oxidation is used up in oxidising a further quantity of the oxidisable substance, nevertheless, in presence of a peroxidase another part of this hydrogen peroxide can bring about a secondary oxidation of various substances such as nitrites and lactic acid. Evidence of the same kind was given in the case of a number of oxidising systems, including those of the sulphhydryl compounds cysteine and glutathione.

It was shown by Abderhalden and Wertheimer [1923] that cysteine is rapidly oxidised to cystine by hydrogen peroxide. These authors used a large excess of hydrogen peroxide in their experiments. In a recent paper, Szent-Györgyi [1926] has shown that a dilute solution of reduced glutathione is oxidised fairly rapidly by excess of hydrogen peroxide. In consequence, he considers that the presence of the sulphydryl group in the tissues will interfere with secondary oxidations brought about in presence of peroxidase by hydrogen peroxide; in other words that peroxidase cannot compete with the oxidising sulphydryl group for a share of the hydrogen peroxide to a sufficient extent to make the secondary oxidations important. This criticism does not appear to be a valid one. In the first place, the conditions in Szent-Györgyi's experiments are not physiological, since excess of hydrogen peroxide is employed and the hydrogen peroxide is all added at once. In the living cell hydrogen peroxide is formed by the primary oxidising systems themselves and is hence produced only in small quantities at a time. If it were present to a greater extent, the cell enzymes, including peroxidase itself, would be destroyed, and the widespread distribution of catalase in the tissues appears to be an additional safeguard against the accumulation of hydrogen peroxide in toxic quantities.

In the second place it is actually possible, as the following experiment will show, for a secondary oxidation by hydrogen peroxide and peroxidase to occur in presence of the sulphydryl group, and even when the hydrogen peroxide is slowly added to the system instead of being formed by the system itself. It was considered worth while to perform this experiment in order to meet Szent-Györgyi's criticism, because the mechanism involved in secondary oxidations brought about by peroxidase and hydrogen peroxide seems too important to be laid aside without due consideration. The following experiment was devised to show that even when hydrogen peroxide is added continuously to a sulphydryl system containing peroxidase together with a suitable oxidisable substance such as a nitrite, the hydrogen peroxide serves not only to oxidise the sulphydryl compound but also, activated by the peroxidase, to bring about the oxidation of the nitrite.

Broadly speaking, the principle of the experiment was slowly to add dilute solutions of cysteine, peroxidase and hydrogen peroxide to a solution of peroxidase containing a small quantity of sodium nitrite, and then to test for the disappearance of the nitrite by the Griess-Ilosvay reagent. The solutions were added in such a way that a small excess of the sulphydryl compound was present in the solution throughout the experiment. Oxidation of the cysteine by the air was entirely prevented by bubbling purified nitrogen through the solution, this also sufficing to keep the solution well stirred.

The cysteine solution containing 13.3 mg. of free cysteine per cc. (neutralised with sodium hydroxide) was quickly poured into a burette, the entry of air into the latter being prevented throughout the experiment by a stream of nitrogen. The peroxidase was prepared from milk by the method of

Thurlow [1925], and a saturated solution in phosphate buffer ( $p_H$  7.6) was used (the solution being filtered and the necessary amount of sodium hydroxide being added to the solution to maintain the  $p_H$  at 7.6). The hydrogen peroxide solution was made up to be equivalent to the cysteine solution. The strength of the hydrogen peroxide solution was checked at the beginning of the experiment by a direct titration of the cysteine solution, a known volume of the latter being made alkaline with ammonium hydroxide, warmed, and titrated with the hydrogen peroxide, using the nitroprusside test as an external indicator.

A solution of 0.1 mg. of sodium nitrite in 20 cc. of peroxidase was contained in a thick glass boiling-tube, a bulb being blown in the upper part of the latter to increase its capacity and to reduce frothing of the protein-containing solution. The tube was fitted with a rubber stopper through which passed an entry and narrow exit tube for nitrogen and three short pieces of glass tubing each drawn out to a fine jet. Three burettes containing the cysteine, hydrogen peroxide and peroxidase respectively, were attached by short pieces of rubber tubing to the three glass tubes the jets of which had previously been adjusted to dip well under the surface of the liquid in the boiling-tube. Before putting in the rubber stopper, the burettes were turned on until all the air in the glass tubes was displaced by liquid. After drying the outside of the tubes with filter paper, the stopper was put into the boiling-tube and a stream of nitrogen (purified by passing through four vessels of pyrogallol) was bubbled through the solution. After all the air had been driven out 1.0 cc. of cysteine was added from the burette, and the tube was immersed in a water-bath at  $37^\circ$ . An excess of cysteine was thus present at the beginning and the excess was maintained throughout the experiment. The cysteine, peroxidase and hydrogen peroxide were now run in from the burettes in successive small quantities in the following order, namely, 0.2 cc. cysteine, 0.5 cc. peroxidase, 0.2 cc. hydrogen peroxide. (The peroxidase was added to replace that which was destroyed during the experiment.) The duration of the experiment was about one and a half hours, and in all 7.5 cc. cysteine, 20.0 cc. peroxidase and 7.5 cc. hydrogen peroxide were added. The tube was now removed from the water-bath and 4.0 cc. of water were added (by removing the nitrogen exit tube), making the total volume of the solution up to 60 cc. In the tests described below, this solution will be called solution B. A fairly rapid stream of nitrogen was maintained to prevent the entry of oxygen, and quantities of the solution were removed for testing by replacing the nitrogen exit tube by a pipette. A test portion of the solution gave a strong nitroprusside test at the end of the experiment, showing that excess of cysteine was present.

In order to test samples of the solution for the disappearance of nitrite, it was necessary first to remove the excess of cysteine, as the latter was found to inhibit the development of the colour in the Griess-Ilosvay test. This was effected by making the solution alkaline and adding hydrogen peroxide, after



destruction of the peroxidase in the solution by heat or by treatment with concentrated hydrochloric acid. (Haas and Lee [1924] have shown that nitrites are not oxidised by hydrogen peroxide in the absence of peroxidase.)

Three tests were carried out for the disappearance of nitrite as follows.

1. A control solution containing 40 cc. peroxidase and 0.1 mg. sodium nitrite was made up to 60 cc. with water. This would represent the concentration of nitrite in the experimental solution if none had been oxidised. 2.5 cc. of this control solution A and 2.5 cc. of the experimental solution B from the boiling-tube were pipetted respectively into two test tubes, each containing 1 cc. of concentrated hydrochloric acid which destroyed the peroxidase. After shaking, the solutions were made alkaline with 1 cc. of concentrated ammonium hydroxide and 0.1 cc. of the hydrogen peroxide solution was added to each to oxidise the cysteine remaining in solution B. (This hydrogen peroxide cannot oxidise any nitrite present since the peroxidase has been destroyed.) After shaking, and confirming the absence of cysteine in B, 2.0 cc. of Griess-Ilosvay reagent were added to each, and the tubes were allowed to stand a few minutes for development of colour. The solution B gave a completely negative test for nitrite, whereas the control solution A gave a definitely positive reaction for nitrite.

2. 2.5 cc. of the control solution A and of the test solution B were taken and the peroxidase in the two solutions was destroyed by boiling. After cooling, 0.1 cc. of ammonium hydroxide and 0.1 cc. of hydrogen peroxide were added. The solutions were well shaken, the coagulated protein being broken up with a glass rod, and 1 cc. of Griess-Ilosvay reagent was added to each. After standing for a few minutes, the control solution A gave a strongly positive test for nitrite, while the experimental solution B gave a completely negative reaction for nitrite.

3. Two quantities of 2.5 cc. of the solution B were pipetted from the boiling-tube and to one was added 0.05 cc. of sodium nitrite solution containing 0.00084 mg. The two solutions were then treated in the manner described in Test 2. The solution, to which 0.05 cc. of nitrite had been added before testing, gave a definitely positive test for nitrite, while the other solution, which had originally contained five times this amount of nitrite, gave a completely negative test. This last test showed that one-fifth of the quantity of nitrite which was originally present in the solution could readily be detected by this method of testing. Further, this last test gives confirmation that the disappearance of nitrite is not due to its oxidation by the hydrogen peroxide added during the test, the peroxidase having previously been destroyed.

The whole experiment was repeated under the same experimental conditions and identical results were obtained.

It is evident, therefore, that hydrogen peroxide in presence of peroxidase brings about the oxidation of nitrites in a solution containing a sulphydryl compound. No attempt was made to find the maximum quantity of nitrite

which could be oxidised by a given quantity of hydrogen peroxide and peroxidase. It was desired merely to show that such an oxidation actually could take place to a measurable extent *in vitro* in the presence of a sulphydryl compound. In our present state of knowledge it is impossible fully to imitate *in vitro* the effects on chemical reactions produced *in vivo* by the specialised structure of the living cell, and such factors as selective permeability and selective adsorption may well play an important part in determining in what proportion the hydrogen peroxide is shared between oxidising sulphydryl compounds and between bringing about, in presence of peroxidase, secondary oxidations in the living cell.

#### SUMMARY.

Experimental evidence is given to show that the presence of sulphydryl compounds does not prevent the oxidation of sodium nitrite by hydrogen peroxide in presence of milk peroxidase.

The bearing of this on secondary oxidations by hydrogen peroxide in the living cell is discussed.

The author wishes to express his sincere thanks to Professor E. Mellanby, F.R.S., for his interest and encouragement.

He also wishes to express his indebtedness for a Keddey Fletcher-Warr Studentship, University of London, held during the course of this work.

#### REFERENCES.

- Abderhalden and Wertheimer (1923). *Pflüger's Arch.* **199**, 336.  
Haas and Lee (1924). *Biochem. J.* **18**, 618.  
Harrison and Thurlow (1926). *Biochem. J.* **20**, 217.  
Szent-Györgyi (1926). *Biochem. Z.* **178**, 75.  
Thurlow (1925). *Biochem. J.* **19**, 175.

# LXXIII. EXPERIMENTS ON THE FORMATION OF SUCCINIC ACID IN THE BODY.

## PART I. THE DETERMINATION OF SUCCINIC ACID AND ITS FORMATION IN MUSCLE AND LIVER PULP.

By PERCIVAL WALTER CLUTTERBUCK.

*From the Department of Physiology, the University, Manchester.*

*(Received April 4th, 1927.)*

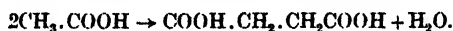
IN several recent publications [*e.g.* Ascher, 1925; Burn and Marks, 1926] considerable support has been given to the view that the liver normally, to some extent, converts fat into reducing sugar, and that this process becomes increasingly important when the animal is deprived of its glycogen supplies. Nothing is yet known, however, of the chemical mechanism of such conversion, nor does the theory of  $\beta$ -oxidation of fats readily provide an obvious mechanism.

Two suggestions have been made. It has been shown [Clutterbuck and Raper, 1926] that acetoacetic acid on oxidation with hydrogen peroxide, which, according to Dakin [1908, 1, 2, 3; 1910], simulates certain physiological oxidations, yields  $\alpha$ -hydroxyacetoacetic acid. It was therefore suggested that in the liver acetoacetic acid, known to arise in the metabolism of fat, might give rise successively to  $\alpha$ -hydroxyacetoacetic acid, diketobutyric acid and finally, by loss of carbon dioxide, to methylglyoxal, which is readily converted by glyoxalase [Dakin and Dudley, 1913, 1914] to lactic acid, and to glucose.

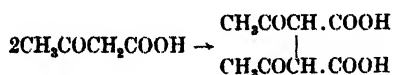
Consideration of the chemistry of muscle leads to the second suggestion. It has been known for a long time [Einbeck, 1913, 1914] that fresh muscle extracts contain considerable amounts of succinic acid, which has not arisen from putrefaction as previously suggested [Wolff, 1903], and the amount in such extracts has recently been determined [Moyle, 1924]. Muscle tissue also contains a very powerful enzyme which acts on succinic acid [Thunberg, 1909; Batelli and Stern, 1911], and is remarkably specific. The enzyme, according to Batelli and Stern, converts succinic acid into optically inactive malic acid, but their identification of the acid was not very convincing. Einbeck [1914] regarded fumaric acid and not malic acid as the sole product, but later [1919] he showed that the enzyme first converted succinic to fumaric acid and then by a balanced reaction to a mixture of fumaric (25 %) and malic (75 %) acids. Dakin [1922] showed that fumaric acid, reacting with muscle

pulp, formed exclusively *laevo*-malic and not the inactive acid, and he pointed out that this asymmetric synthesis was of additional interest from the fact of the stereochemical similarity of *l*-malic and *d*-lactic acids. The succinic acid formed *in vivo* may, therefore, represent an intermediate stage of a special type of sugar formation by way of fumaric, malic and lactic acids. It is known that malic acid yields glucose in the diabetic organism and that succinic acid gives "extra glucose" in phloridzinised animals [Ringer, Frankel and Jones, 1913].

Nothing is known as to the origin of succinic acid in the body. Although succinoxidase is so very powerful, specific and widely distributed, yet its only function appears at the present time to be that of dealing with any small amounts of succinic acid supposed to arise from glutaminic acid during the metabolism of proteins. It is interesting to enquire whether succinic acid might not arise by direct oxidation of fatty acids in the body. During oxidations *in vitro* with hydrogen peroxide, formation of succinic acid very often occurs. Thus butyric acid [Cahen and Hurlley, 1917] gives, besides acetoacetic acid, a considerable amount of succinic acid. Also caproic, oenanthic, caprylic, myristic, palmitic and stearic acids [Clutterbuck and Raper, 1925] give, besides the  $\beta$ -oxidation product,  $\gamma$ -keto-acids; and the first three,  $\delta$ -keto-acids also. These on further oxidation form succinic acid. Thunberg has also suggested that acetic acid may yield succinic acid *in vivo*



Knoop and Gehrke [1925] oxidised acetic acid and acetone by standing at the ordinary temperature for a long time with hydrogen peroxide, and although but little oxidation of acetic acid occurred, they obtained from acetone considerable amounts of a mixture of tartaric, malic and succinic acids. It has also been shown [Clutterbuck and Raper, 1926] that acetoacetic acid gives on oxidation with hydrogen peroxide, besides  $\alpha$ -hydroxyacetoacetic acid, a considerable amount of acetylacetoacetic acid and a little ethyl isocarbo pyrotritartrate, and it was suggested that these had arisen from the intermediate formation of diacetylsuccinic acid. It was considered that possibly diacetylsuccinic acid might similarly arise from acetoacetic acid *in vivo*



and give rise on hydrolysis to succinic acid.

Kay and Raper [1922] injected atropic acid into dogs and isolated a small amount of succinic acid from the urine. It would seem almost impossible that this acid could have been formed from the substance injected. Atropic acid, however, on injection caused some damage to the kidney, as evidenced by haemoglobinuria, and the explanation of the appearance of succinic acid possibly lies in this fact. This question is being further investigated.

The above evidence has led the author to embark upon a study of the

formation of succinic acid with the object of throwing some light on its origin. The method of Moyle [1924] gives excellent results with very small amounts of the acid. It is, however, rather long and tedious, and is not suitable for our purpose on account of the extremely great activity of succinoxidase, which is capable of disposing of large amounts of succinic acid in a short time. It seemed better, therefore, to attempt to follow the production of this acid not directly but in terms of the *l*-malic acid arising from it by the action of the enzyme, the malic acid being not readily further attacked in experiments with the isolated tissue and being rapidly determinable by means of its optical rotation. In the present paper, a polarimetric method of this kind is described. It has been used to follow the conversion of succinic and fumaric acids to malic acid, and the effect on these reactions of the addition of cyanide has been determined. It has also been adapted to the detection of smaller amounts of succinic acid and, in order to test the above suggestions, it was applied to determine whether succinic acid was produced in liver and muscle pulp when the following substances were added: sodium acetate, acetoacetate,  $\alpha$ -ketoglutarate,  $\delta$ -ketoheptate, diacetylsuccinate and acetone. It was found that although liver and muscle, under the conditions of the experiments, were able very readily to oxidise (*i.e.* dehydrogenate) succinic acid, yet they were not able to oxidise any of the above substances in the way that hydrogen peroxide could do. Thus sodium acetate was not attacked, acetone, acetoacetic and diacetylsuccinic acids did not give rise to succinic acid,  $\delta$ -ketoheptic acid, which was obtained by oxidation of heptic acid with hydrogen peroxide and which very readily gave succinic acid on oxidising further, did not give an amount detectable by the method, and  $\alpha$ -ketoglutaric acid, which may be expected to yield succinic acid in the intact animal, did not do so in these experiments. The *l*-malic acid, formed from succinic acid, also remains as such, although in the intact animal it is supposed to lose carbon dioxide yielding lactic acid, since it yields glucose in the diabetic animal. Similar differences between results under experimental conditions and in the intact animal are found when the liver is perfused. Raper and Smith [1926] found that only 80 % of the theoretical acetone could be recovered from perfused butyric acid, and concluded that the liver oxidised the remaining 20 % without the appearance of acetone bodies. On the other hand, Snapper and Greenbaum [1927] found that when either acetoacetic or  $\beta$ -hydroxybutyric acid was perfused, the latter was absorbed by the liver tissue, the amount contained by the liver being sometimes as great as six to eight times the amount expected from the acetone content of the blood. Taking this absorption into account, the amount of these acids further oxidised is very small indeed. The perfused liver, however, in that it has been shown to attack a large number of amino-acids, does appear to be much more powerfully oxidising than the isolated liver without its blood supply. It is proposed therefore to investigate the effect of perfusion of some of the substances referred to above through the surviving liver.

## EXPERIMENTAL.

The immediate purpose of the experiments was to find out what substances, on addition to liver and muscle pulp, could result in the formation of succinic acid. Such substances, incubated with the pulp in presence of oxygen, should give rise to *l*-malic acid and be determinable, therefore, in virtue of the resulting change in rotation. In order to decide whether the formation of succinic acid could be detected in this way, a method was first devised for following the changes in rotation obtained when succinic acid is added to liver and muscle pulp.

*Detection of succinic acid, added to muscle and liver pulp.*

The method is an adaptation of that used by Dakin [1922] with fumaric acid, and depends on the fact that the normal small rotation of *l*-malic acid in water,  $[\alpha]_D^{20} - 1.7^\circ$ , is increased in presence of uranium acetate to  $[\alpha]_D^{20} - 482^\circ$ . The muscle and liver used in these experiments were obtained from rabbits under sterile conditions. After killing the animal, the fur near the line of incision was removed, the animal washed with water and lysol, the skin carefully retracted and clipped back, and the back and leg muscles excised, using sterilised instruments. The dishes, mincer, container and pipettes were also sterilised. In each experiment, 5 g. of succinic acid were neutralised to litmus, diluted to 500 cc. and added to 100 g. minced sterile muscle or to 30 g. minced liver, from which the gall bladder and main portal tracts had been removed before mincing. The material was placed in a sterilised bottle (capacity about 1200 cc.) and oxygenated by passing in the gas through a cotton wool plug and sterilised tube, until the whole of the air was displaced with oxygen. The bottle was then closed with a rubber bung, wired and rotated in a thermostat at  $38^\circ$ . It was opened hourly for re-oxygenation and removal of samples. Each sample (25 cc.) was heat-coagulated on a boiling water-bath, 10 g. solid uranium acetate were added, made up to 100 cc., cooled, filtered and transferred to a polarimeter tube (4 decimetre) and the rotation determined. If the filtered solution was allowed to stand, occasionally a very slight opalescence appeared which could not be removed by filtration and which made it difficult to obtain a reading. Such solutions on standing longer, especially in sunlight, deposited a small amount of a flocculent precipitate and on filtration were clear. In two experiments also in which much larger amounts of a liver containing a considerable amount of glycogen were used, the glycogen opalescence made readings difficult.

The method was first applied to the incubated pulp without addition of sodium succinate. The zero reading was quite constant for different samples of muscle, and the change in rotation during 6 hours' incubation either nil or slightly dextro ( $+ 0.2^\circ$ ).

The changes in rotation in one of a number of experiments in which

5 g. succinic and 5 g. fumaric acids (as sodium salts) respectively had been added to 100 g. minced sterile muscle were as follows:

Muscle sample	5 g. succinic acid added			5 g. fumaric acid added		
	Time hours	Rotation	<i>l</i> -Malic acid formed (g.)	Time hours	Rotation	<i>l</i> -Malic acid formed (g.)
1	0	0.0 °	0.0	0	0.0 °	0.0
2	1	-0.30	0.36	1	-1.22	1.46
3	2	-0.63	0.75	2	-1.96	2.34
4	3	-0.88	1.05	3	-2.64	3.15
5	4	-1.18	1.41	4	-2.82	3.36
6	5	-1.42	1.69	5	-2.90	3.46
7	6	-1.64	1.96	6	-2.90	3.46
8	7	-1.83	2.18			
9	8	-2.01	2.40			
10	10	-2.36	2.82			
11	11	-2.40	2.86			
12	12	-2.40	2.86			
13	13	-2.40	2.86			

In calculating the *l*-malic acid formed, a small correction was applied for the volume of water contained respectively by the muscle and liver.

Replacing muscle by 30 g. liver, the following results were obtained:

Liver sample	5 g. succinic acid added			5 g. fumaric acid added		
	Time hours	Rotation	<i>l</i> -Malic acid formed (g.)	Time hours	Rotation	<i>l</i> -Malic acid formed (g.)
1	0	0.0 °	0.0	0	0.0 °	0.0
2	1	-0.59	0.64	0.5	-1.53	1.65
3	2	-1.22	1.32	1.0	-2.46	2.65
4	3	-1.90	2.05	1.25	-2.84	3.06
5	4	-2.58	2.79	1.50	-3.02	3.26
6	5	-3.12	3.37	1.75	-3.20	3.45
7	6	-3.28	3.54	2	-3.27	3.52
8	7	-3.28	3.54	3	-3.27	3.52

The experiments with 100 g. sterile muscle and 5 g. succinic and fumaric acids (as sodium salts) were then repeated with sufficient potassium cyanide added to make the solution 0.03 % KCN. The results are as follows:

Muscle sample	5 g. succinic acid added			5 g. fumaric acid added		
	Time hours	Rotation	<i>l</i> -Malic acid formed (g.)	Time hours	Rotation	<i>l</i> -Malic acid formed (g.)
1	0	0.0 °	0.0	0	0.0 °	0.0
2	2	-0.62	0.74	1	-1.22	1.46
3	3	-0.64	0.76	2	-1.94	2.31
4	4	-0.64	0.76	3	-2.58	3.08
5	6	-0.64	0.76	4	-2.79	3.32
6	7	-0.64	0.76	5	-2.84	3.38
7	—	—	—	6	-2.84	3.38

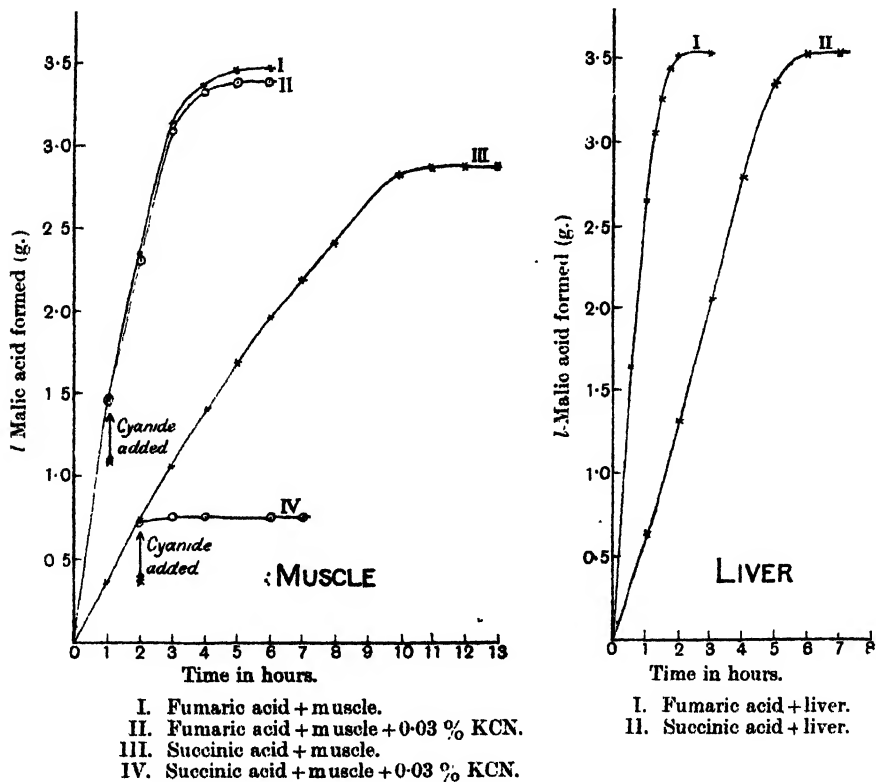
The cyanide was added, in the succinic acid experiment, after 2 hours and, in the fumaric acid experiment, after 1 hour had elapsed from the beginning of the experiment.

When these results are plotted, a number of interesting observations may be made.

(1) Rabbit liver, weight for weight, is about eight times as active as rabbit muscle in the conversion of *both* succinic *and* fumaric acids to malic acid.

(2) The power of the liver enzyme to attack succinic and fumaric acids is remarkably great, 30 g. of liver converting 5 g. of succinic acid to the equilibrium mixture in 6 hours, and 5 g. fumaric acid in 2 hours. This will render isolation of these acids in the body an extremely difficult task. The velocity of the oxidation reaction is obviously much less than that of the subsequent addition of water and not *vice versa*, as has often been supposed.

(3) The succinic acid reaction with muscle does not attain quite to the same equilibrium as the fumaric acid reaction. This is probably due to the longer time required in the former, with resulting onset of bacterial infection, which often begins to make itself evident after 10–12 hours.



(4) Cyanide (0.03 %) completely stops the succinic acid reaction but has no effect on the fumaric acid reaction. The latter is therefore a simple addition of the elements of water and not an oxido-reduction, and the action of cyanide is strictly localised to the oxidation phase.

The curves above are readily reproducible with different samples of muscle and liver, the slight differences affecting the equilibrium point rather than direction of the curves.



*Isolation and identification of the l-malic acid produced.*

The *l*-malic acid formed by the oxidation of succinic acid with tissue pulp was isolated and identified in the following way. The resulting oxidation mixture was heated in a boiling water-bath to coagulate the proteins and filtered through muslin. The coagulated protein was washed twice in hot water and again filtered through muslin. The combined filtrate and washings were then filtered through papers and the filtrate evaporated under reduced pressure to about 200 cc., a concentrated solution of lead acetate (20 g.) added and then a little ammonia until the liquid was just alkaline, and finally about one-third the volume of alcohol, and the whole allowed to stand overnight. The precipitate containing malic and fumaric acids was filtered off, washed with a little cold water, decomposed with a slight excess of sulphuric acid and filtered. The filtrate was made alkaline with barium hydroxide and the precipitated phosphate and fumarate filtered off. The filtrate was then neutralised, the malic acid precipitated as the silver salt and recovered by passing hydrogen sulphide into its suspension in water, filtered and evaporated. The crude acid was then dissolved in a little water and extracted with butyl alcohol under reduced pressure by Dakin's [1920] method. For final identification, the *l*-malic acid was converted into the cinchonine salt, m.p. 197–198°, and shown to be identical with a synthetic sample obtained by resolving inactive malic acid by means of cinchonine [Dakin, 1924]. The mixed melting point and rotations were identical with those obtained by Dakin.

*Limits of the method.* In the preceding experiments, a 1 % solution of succinic and fumaric acids had been used. It was next attempted to find out if the presence of much smaller amounts of succinic acid could be detected by this rotational method. In this series of experiments, 100 g. muscle or 30 g. liver were added to 500 cc. of a neutralised 0.1 % solution of succinic and fumaric acids, and, for heat-coagulation, a sample of 100 cc. was removed, heat-coagulated, uranium acetate added and the rotation found as before. The following are typical results with muscle:

Acid	Time (hours)	Change in rotation	<i>l</i> -Malic acid formed (g.)
Succinic	6	– 0.90°	0.27
Fumaric	6	– 1.12	0.33

These results obtained with 0.1 % solutions of the acids compare very well with the equilibrium values obtained previously with 1 % solutions, for succinic acid 2.86, and for fumaric acid, 3.46. The above figures show that although the method has only been used for detection of amounts of succinic acid of this order, yet it is capable of greater delicacy by taking larger samples or by using smaller changes in rotation.

*Application of the method.* The method, in the adapted form, was then used in attempts to detect the formation of succinic acid in liver and muscle pulp from the following possible precursors, viz. acetone, sodium acetate,  $\alpha$ -ketoglutarate, acetoacetate, diacetylsuccinate, and  $\delta$ -ketoheptate. The sub-

stances in 500 cc. of water were added to the muscle or liver pulp (in place of the succinate) and rotated in oxygen for 6 hours, reoxygenating hourly. The difference in optical rotation at the beginning and end of the period was in every case slightly dextro (as in a blank determination). It would appear, therefore, that although the above substances (except diacetylsuccinate) have been shown to yield succinic acid with hydrogen peroxide, they are either not similarly oxidised by muscle and liver pulp under the conditions of these experiments, or the amount of succinic acid formed is so small that it cannot be detected by the method.

*Preparation of  $\delta$ -ketohezoic acid.* [See Clutterbuck and Raper, 1925.]

*Preparation of sodium diacetylsuccinate.* Ethyl diacetylsuccinate was first obtained by the action of iodine on ethyl sodioacetoacetate in ether solution by the method of Rugheimer [1874], and converted into ethyl isocarbopyrotritartrate by the method of Knorr and Haber [1894], 10 g. of ethyl diacetylsuccinate yielding only 3.85 g. of the recrystallised product. This substance was converted into sodium diacetylsuccinate just before use.

Ethyl isocarbopyrotritartrate (6.8 g.) was warmed with 54.4 g. of a 20 % solution of sodium hydroxide until the solution became clear, and boiled for  $6\frac{1}{2}$  minutes. It was cooled under the tap and in iced water, and then added to 13.6 cc. of sulphuric acid in 102 cc. of water. The free isocarbopyrotritartronic acid was filtered off, washed, dried and dissolved in the theoretical amount of sodium hydroxide by heating for 1 minute. The sodium diacetylsuccinate thus formed was made up to 500 cc. and added to the liver pulp as before.

*Preparation of  $\alpha$ -ketoglutaric acid.* By condensation of ethyl oxalate and succinate in presence of sodium ethoxide, an ester is obtained [Wislicenus, 1889], which according to Blaise and Gault [1908], on boiling with hydrochloric acid and evaporating, yields  $\alpha$ -ketoglutaric acid. On attempting to obtain the acid by this method, by evaporating on the water-bath, a viscous syrup was obtained which, as Gabriel [1909] showed, readily reacts with hydrazine and must contain a considerable amount of  $\alpha$ -ketoglutaric acid; yet it was difficult to obtain a pure specimen by recrystallisation. The method of obtaining the pure acid was as follows. Ethyl oxalylsuccinate was first prepared by the method of Wislicenus and Waldmüller [1911]. Potassium (13.4 g.) was added to a mixture of absolute, dry ether (150 g.) and absolute alcohol (40 g.) distilled over sodium and the whole warmed under a reflux until dissolved and then cooled. Pure oxalic ester (50 g.) was then added and after 10 minutes pure succinic ester (59.5 g.) poured in with shaking. The potassium salt of the double ester began to separate immediately and after standing a few hours the solution had set to a solid mass. The ether-alcohol and any unchanged ester were squeezed out of the solid mass by means of a press, the solid again shaken with ether and the ether similarly removed.

The potassium salt was then suspended in water, acidified and the ester

extracted with ether, the ethereal solution dried over sodium sulphate, and the ether removed. The ester was then boiled for 2 hours under reflux with six times its weight of diluted hydrochloric acid (1 in 2) and evaporated *in vacuo* (1 mm.) from a water-bath completely to dryness, when almost pure  $\alpha$ -ketoglutaric acid set solid in the distilling flask. If the evaporation is not carried out *in vacuo*, other products, probably of condensation, arise and cause the difficulty in obtaining a pure product by recrystallisation. The product was dissolved in a little warm acetic acid and allowed to stand. After several hours, a little acid had separated and this was filtered off and the solution stirred and allowed to stand overnight. The bulk of the acid now separated and this second crop was practically pure and melted at 110–112°. It was once more recrystallised from acetic acid and melted at 112°. It readily gave a semicarbazone, M.P. 220°.

#### SUMMARY.

(1) A polarimetric method for following the conversion of succinic acid to fumaric and malic acids is described.

(2) Using the minced organs, rabbit liver is eight times as active, weight for weight, as rabbit muscle in bringing about the conversion *both* of succinic to fumaric acid and of fumaric to malic acid.

(3) Using the method, with the minced organs, no production of succinic acid from the following possible precursors could be detected: acetone, sodium acetate, acetoacetate,  $\alpha$ -ketoglutarate,  $\delta$ -ketohexonate and diacetylsuccinate.

(4) Cyanide, while inhibiting completely the change from succinic to fumaric acid, has no action on the conversion of fumaric to malic acid. The latter change is therefore not an oxido-reduction process but a simple addition of the elements of water.

In conclusion, the author desires to thank Professor H. S. Raper for helpful suggestions and criticism, and Mr O. N. Jones for considerable assistance in the experimental part of this work.

#### REFERENCES.

- Ascher (1925). *Biochem. Z.* **164**, 76.  
Batelli and Stern (1911). *Biochem. Z.* **30**, 172.  
Blaise and Gault (1908). *Compt. Rend. Acad. Sci.* **147**, 198.  
Burn and Marks (1926). *J. Physiol.* **61**, 497.  
Cahen and Hurlley (1917). *Biochem. J.* **11**, 164.  
Clutterbuck and Raper (1925). *Biochem. J.* **19**, 385.  
—— (1926). *Biochem. J.* **20**, 59.  
Dakin (1908, 1, 2, 3). *J. Biol. Chem.* **4**, 77, 221, 227.  
—— (1910). *Amer. Chem. J.* **44**, 41.  
—— (1920). *J. Biol. Chem.* **44**, 499.  
—— (1922). *J. Biol. Chem.* **52**, 183.  
—— (1924). *J. Biol. Chem.* **61**, 139.  
Dakin and Dudley (1913). *J. Biol. Chem.* **14**, 155, 423.

- Dakin and Dudley (1914). *J. Biol. Chem.* **15**, 463.  
Einbeck (1913). *Z. physiol. Chem.* **87**, 145.  
—— (1914). *Z. physiol. Chem.* **90**, 301.  
—— (1919). *Biochem. Z.* **95**, 296.  
Gabriel (1909). *Ber. deutsch. chem. Ges.* **42**, 655.  
Kay and Raper (1922). *Biochem. J.* **16**, 472.  
Knoop and Gehrke (1925). *Z. physiol. Chem.* **146**, 63.  
Knorr and Haber (1894). *Ber. deutsch. chem. Ges.* **27**, 1151.  
Moyle (1924). *Biochem. J.* **18**, 351.  
Raper and Smith (1926). *J. Physiol.* **62**, 30.  
Ringer, Frankel and Jones (1913). *J. Biol. Chem.* **14**, 539.  
Rugheimer (1874). *Ber. deutsch. chem. Ges.* **7**, 892.  
Snapper and Greenbaum (1927). *Biochem. Z.* **181**, 410.  
Thunberg (1909). *Skand. Arch. Physiol.* **22**, 430.  
Wislicenus (1889). *Ber. deutsch. chem. Ges.* **22**, 885.  
Wislicenus and Waldmüller (1911). *Ber. deutsch. chem. Ges.* **44**, 1564.  
Wolf (1903). *Beitr. chem. Physiol. Path.* **4**, 254.

# LXXIV. STUDIES OF THE UREA CONTENT OF CAPILLARY AND VENOUS BLOOD.

By ELISABETH SVENSGAARD.

*From the Medical Department of the County Hospital, Aarhus, Denmark.*

*(Received April 7th, 1927.)*

THE question as to whether equilibrium exists between the concentrations of urea in the blood and those of the various organs and secretions has been studied by Gad-Andresen [1921]. He arrived at the conclusion that equilibrium exists as far as the secretions are concerned, with the exception of tears and sweat, where the urea content increases with increased secretion. As for the organs, Gad-Andresen's investigations are not conclusive. In muscle he finds a lower urea content than in blood, whereas the ammonia content is higher in such a proportion that the sums of the two substances are identical for muscle and blood. In the liver the concentration of urea is found to be the same as in the blood, while in fatty tissue it is less. For his determinations Gad-Andresen used a micro-method devised by himself.

Tschudi-Madsen [1924], using Van Slyke's urease method for his studies on the distribution of urea, found in all cases higher concentrations of urea in the organs than in the blood. The values for urea in exudates, transudates and cerebrospinal fluid were found to lie very close to those for serum, though slightly lower on an average.

Neither of these authors gives any data with regard to the distribution of urea in arterial and venous blood. This question has been studied by some other investigators, from whose works the following data may be quoted [from Bollman, Mann and Magath, 1924]: Pousseule and Gobley found in some cases a higher, in some cases a lower urea content in venous than in arterial blood; Picard found in some cases equal values, in some cases higher values for venous blood; Grehaut and Quincaud found equal values as far as the blood of the extremities was concerned, whilst venous blood derived from the liver and spleen contained more urea than arterial blood from the same organs. Kauffmann did not find any difference.

## I. TECHNIQUE.

For my determinations I first tried the micro-method devised by Göthgen [1925]. However, as the results showed too great variations, I resorted to that of Rehberg [1925, 2]. My technique was as follows: 0.5 cc. of distilled water is placed in a test-tube and 0.1 cc. blood, measured in a Hagedorn

pipette, is added. The pipette is washed through three times with the distilled water. Then 0.1 cc. of a fresh 10 % urease solution (Squibb), adjusted to  $p_H$  7 with a phosphate buffer solution, is added. The tube, being closed with a rubber plug, is placed on the water-bath at 40° for 30 mins. Then three drops of octyl alcohol and 1 cc. saturated  $K_2CO_3$  solution are added. Previously, however, 2 cc.  $N/600$   $H_2SO_4$  solution, freshly prepared from  $N/10$  solution, should be measured into each titration tube, one drop of octyl alcohol being added. The test-tubes and titration tubes are then connected and aeration takes place for 2.5 hours at a speed moderate enough to avoid spraying the mixture over the walls of the tubes, which gives rise to inaccuracy. One or two blank analyses are set up, and a control with a known urea solution. For the control a 60 mg. % urea solution was used.

When aeration is completed the solution is titrated with  $N/30$  NaOH by means of Rehberg's microburette [1925, 1]. Methylene red as indicator was used in a 0.05 % solution. The mean error in my tests was  $\pm 1$  mg. urea.

The blood samples were drawn in the morning, while the persons were still in bed and fasting; only in a few cases had they been out of bed for about an hour. The capillary blood was drawn from the ear after it had been rendered slightly hyperaemic by rubbing. Three or four samples were taken in immediate succession with a Hagedorn pipette, which was rinsed in a 3 % citrate solution before each drawing. The venous blood was taken from an arm vein, either without stasis or after a slight stasis had been procured, after I had ascertained that this made no difference to the results. The blood was evacuated into a dry test-tube, from which a number of tests, corresponding to those of capillary blood, were immediately measured off. Addition of anticoagulants proved unnecessary. The tubes were then placed in the ice-box until analysis could be undertaken a few hours later. It was experimentally substantiated that standing did not influence the constancy of the results provided the test-tubes were kept directly surrounded by ice.

## II. NORMAL INDIVIDUALS.

(a) *Fasting values.* I have performed analyses with blood from 18 normal persons, altogether 32 sets of analyses with capillary and venous blood. The majority of the persons tested were convalescents after some mild affection not involving metabolism.

In 27 of these 32 cases the urea concentration was found to be higher in capillary than in venous blood, with an average of 10 %. The percentage difference varied, somewhat, from time to time in the same individual as well as from one individual to another. Differences of 4 to 22 % were found. The absolute difference was on an average 2-3 mg., the lowest value being 0.9 mg. and the highest 5.7 mg.

Only in five cases out of the 32 did the venous blood show a higher urea concentration than the capillary blood, the difference being here 1 to 2 % except in a single case, where it was 20.4 % (Nos. 4 c, 5 c, 9, 12 c and 15 b,

Table I). The examination was repeated several times in four of the subjects showing the latter relation, and it appeared that on other occasions these subjects followed the main rule: higher concentration in capillary than in venous blood. The analysis which presented a much higher concentration in venous blood (20.4 %) was one in a set performed during 6 hours of fasting with the purpose of determining the urea level during this period. It appeared that there were only slight variations of the urea concentration. At the beginning and termination of the experiment the venous blood was also examined. The initial sample of venous blood had, as stated, a higher urea concentration than the capillary blood, whilst the final sample of venous blood showed a lower concentration of urea than the capillary blood drawn simultaneously (14 %).

Table I. *Normal individuals.*

No.	Sex	Age	Urea content, mg. per 100 cc.		Difference	
			Capillary blood	Venous blood	mg.	Per cent. in relation to lowest value
1	♂	12	31.6	27.6	4.0	14.4
2	♀	22	35.5	32.7	2.8	8.5
3a	♀	23	27.8	25.0	2.8	11.2
3b	♀	"	32.8	29.8	3.0	10.0
4a	♂	24	20.1	19.2	0.9	4.6
4b	♀	"	24.0	21.0	3.0	14.2
4c	♀	"	22.7	23.3	0.6	2.6
5a	♀	26	16.8	14.3	2.5	17.4
5b	♀	"	17.8	16.8	1.0	5.9
5c	♀	"	19.4	19.7	0.3	1.5
6	♀	27	36.1	33.7	2.4	7.1
7	♀	29	26.7	24.1	2.6	10.7
8	♂	"	27.5	24.2	3.3	13.6
9	♀	"	28.5	29.0	0.5	1.7
10	♀	30	37.9	33.5	4.4	13.1
11	♂	31	40.5	37.3	3.2	8.5
12a	"	34	31.5	30.0	1.5	5.0
12b	"	"	31.5	30.1	1.4	4.6
12c	"	"	23.0	27.7	4.7	20.4
13a	"	35	22.8	18.6	4.2	22.5
13b	"	"	40.7	35.0	5.7	16.2
14	"	36	29.5	25.4	4.1	16.1
15a	"	40	38.9	36.5	2.4	6.5
15b	"	"	39.1	39.9	0.8	2.0
15c	"	"	42.1	38.9	3.2	8.2
15d	"	"	43.8	41.4	2.4	5.7
15e	"	"	47.2	44.3	2.9	6.5
15f	"	"	48.8	45.5	3.3	7.2
15g	"	"	54.3	50.2	4.1	8.1
16	"	42	36.9	33.3	3.6	10.8
17	"	"	42.0	38.0	4.0	10.5
18	"	45	46.2	42.3	3.9	9.2

(b) *Results after ingestion of various substances.* In two normal persons I undertook a series of analyses after the ingestion of various substances. In these cases the blood samples were taken from the ear at an hour's interval, venous blood being drawn two or three times during the course of the experiment.

In one person the substances ingested were (1) urea, 15 g., (2) protein, 35 g., (3) a diet poor in protein, consisting of French beans, butter, coffee with cream, and some rusks.

The fasting blood-urea in this person ranged on an average somewhat higher than the usual value for normals; he was however apparently a perfectly healthy man with normal urine and normal blood-pressure.

With another person analyses were undertaken after the ingestion of 70 g. fat + 70 g. starch, and after 70 g. glucose.

Following the ingestion of 15 g. urea the blood-urea showed a rise from 48 to 74 mg. in the course of 2 hours. After 10 hours the curve had not fallen to the initial level. In these cases the urea content of the capillary blood was 5-7 % higher than that of the venous blood.

Table II. 15 g. urea test.

	♂ 40 years. (No. 15.)		Difference	
	Urea content, mg. per 100 cc.		mg.	Per cent. in relation to lowest value
	Capillary blood	Venous blood		
Before intake	48.8	45.5	3.3	7.2
1 hr. after intake	69.1	—	—	—
2 hrs.     "	74.5	—	—	—
3     "     "	69.4	—	—	—
4     "     "	72.1	67.0	5.1	7.6
5     "     "	68.8	—	—	—
6     "     "	68.6	—	—	—
7     "     "	62.8	59.7	3.1	5.1
8     "     "	59.3	—	—	—
9     "     "	57.4	—	—	—
10   "     "	60.6	—	—	—

After ingestion of 35 g. protein the urea concentration rose in the course of 5 hours from 39 to 46 mg., falling again to the initial value in the course of 10 hours. The urea content of the capillary blood was from 1 to 6 % higher than that of the venous blood (Table III).

Table III. 35 g. protein test.

	Normal individual. ♂ 40 years. (No. 15.)		Difference	
	Urea content, mg. per 100 cc.		mg.	Per cent. in relation to lowest value
	Capillary blood	Venous blood		
Before intake	38.9	36.5	2.4	6.5
1 hr. after intake	39.1	—	—	—
2 hrs.     "	38.6	—	—	—
3     "     "	39.6	—	—	—
4     "     "	43.3	42.8	0.5	1.1
5     "     "	46.1	—	—	—
6     "     "	41.9	—	—	—
7     "     "	41.5	40.1	1.4	3.4
8     "     "	38.4	—	—	—
9     "     "	37.6	—	—	—
10   "     "	38.4	—	—	—



The results obtained after the person had been on a diet poor in protein (Table IV) showed only slight variations in the blood-urea content, just as was the case after ingestion of glucose (Table V) and fat + starch (Table VI).

The higher value for urea was found in the capillary blood except towards the close of the experiment with glucose, where the venous blood contained 3.4 % more urea than the capillary blood. The experiments thus confirmed the findings with regard to fasting values, the main rule being that the higher concentration of urea is found in capillary blood.

Table IV. *Diet poor in protein.*

	♂ 40 years. (No. 15.)		Difference	
	Urea content, mg. per 100 cc.		mg.	Per cent. in relation to lowest value
	Capillary blood	Venous blood		
Before intake	54.3	50.2	4.1	8.1
1 hr. after intake	52.9	—	—	—
2 hrs. "	52.6	—	—	—
3 " "	52.4	—	—	—
4 " "	51.6	49.1	2.5	5.0
5 " "	50.3	—	—	—
6 " "	51.2	—	—	—
7 " "	50.1	49.9	0.2	0.4
8 " "	48.8	—	—	—
9 " "	51.5	—	—	—
10 " "	49.1	—	—	—

Table V. *70 g. glucose test.*

	♂ 34 years. (No. 12.)		Difference	
	Urea content, mg. per 100 cc.		mg.	Per cent. in relation to lowest value
	Capillary blood	Venous blood		
Before intake	25.6	25.5	0.1	—
1 hr. after intake	30.1	—	—	—
2 hrs. "	28.8	—	—	—
3 " "	31.1	—	—	—
4 " "	30.5	—	—	—
5 " "	30.7	—	—	—
6 " "	29.1	30.1	1.0	3.4

Table VI. *70 g. fat + 70 g. starch test.*

	♂ 34 years. (No. 12.)		Difference	
	Urea content, mg. per 100 cc.		mg.	Per cent. in relation to lowest value
	Capillary blood	Venous blood		
Before intake	31.5	30.0	1.5	5.0
1 hr. after intake	28.9	—	—	—
2 hrs. "	28.7	—	—	—
4 " "	29.5	—	—	—
5 " "	29.7	—	—	—
6 " "	28.5	26.3	2.2	8.3

## III. PATHOLOGICAL CASES.

(a) *Fasting values.* I examined three groups of patients in whom one might expect to have found deviations from the normal with regard to the blood-urea relations. They were: 8 cases of icterus, 5 cases of various forms of nephritis and 9 diabetics.

Seven out of the eight analyses of icterus cases showed from 2 to 23 % more urea in capillary than in venous blood and only in one case was the value slightly higher for the venous blood. (No. 22, Table VII, 0.8 %.)

Table VII. *Icterus.*

No.	Sex	Age	Urea content, mg. per 100 cc.		Difference		Diagnosis
			Capillary blood	Venous blood	mg.	Per cent. in relation to lowest value	
19 a	♂	19	25.2	23.9	1.3	5.4	Icterus catarrhalis
19 b	"	"	29.0	24.7	4.3	17.4	"
19 c	"	"	33.6	30.1	3.5	11.6	"
20 a	"	20	25.1	23.6	1.5	6.3	"
20 b	"	"	27.1	26.5	0.6	2.2	"
21	♀	43	12.3	10.0	2.3	23.0	"
22	♂	63	33.9	34.2	0.3	0.8	Cholelithiasis
23	"	66	25.9	23.8	2.1	8.8	"

The five cases of nephritis, some of which had a considerably increased concentration of blood-urea, all showed higher values for the capillary blood.

Table VIII. *Renal diseases.*

No.	Sex	Age	Urea content mg. per 100 cc.		Difference		Diagnosis
			Capillary blood	Venous blood	mg.	Per cent. in relation to lowest value	
24	♂	8	20.2	19.2	1.0	5.2	Glomerulonephritis
25	"	"	77.8	74.1	3.7	4.9	"
26	"	12	55.9	49.4	6.5	13.1	"
27	"	22	52.3	52.2	0.1	0.2	Nephrosis
28	♀	56	103.0	101.9	1.1	1.0	Nephrosclerosis

Eight of nine diabetics followed the main rule; only in one case the reverse relation was found. (No. 30, Table IX.)

Table IX. *Diabetics.*

No.	Sex	Age	Urea content, mg. per 100 cc.		Difference	
			Capillary blood	Venous blood	mg.	Per cent. in relation to lowest value
29	♀	24	32.2	31.5	0.7	2.2
30	♂	39	35.7	36.3	0.6	1.6
31	"	48	44.2	42.0	2.2	5.2
32	"	49	34.9	31.1	3.8	12.2
33	"	"	23.7	22.6	1.1	4.8
34	♀	55	25.4	24.6	0.8	3.2
35	♀	58	26.3	21.2	5.1	24.0
36	♀	60	87.3	83.2	4.1	4.9
37	♀	66	24.7	23.1	1.6	6.9

(b) *Results after ingestion of various substances.* One of the icterus cases was examined after the intake of 35 g. protein and 15 g. urea. The curves resembled very much those found for normals: an abrupt rise following the ingestion of urea (Table XI) and a slow rise after the ingestion of protein with maximum after 5 hours (Table X). In these cases also the main rule was followed with one single exception (Table X, 3 hours after ingestion).

Table X. 35 g. protein test.

		Icterus, ♂ 19 years. (No. 19.)		Difference	
		Urea content, mg. per 100 cc.			
		Capillary blood	Venous blood	mg.	Per cent. in relation to lowest value
Before intake		25.2	23.9	1.3	5.4
1 hr. after intake		28.9	—	—	—
2 hrs.	"	29.7	—	—	—
3 "	"	28.1	28.4	0.3	1.0
5 "	"	32.8	—	—	—
6 "	"	29.0	27.1	1.9	7.0
8 "	"	27.1	—	—	—

Table XI. 15 g. urea test.

		Icterus, ♂ 19 years. (No. 19.)		Difference	
		Urea content, mg. per 100 cc.			
		Capillary blood	Venous blood	mg.	Per cent. in relation to lowest value
Before intake		29.0	24.7	4.3	17.4
1 hr. after intake		66.2	—	—	—
2 hrs.	"	55.7	—	—	—
4 "	"	49.2	47.6	1.6	3.3
6 "	"	43.4	—	—	—
8 "	"	52.0	—	—	—
10 "	"	45.9	43.2	2.7	6.2

## SUMMARY.

Twenty-seven out of 32 sets of analyses of blood from 18 fasting normal individuals showed a higher concentration of urea in capillary blood (from ear-lobe) than in venous blood (from vena mediana cubiti), 10 % higher on an average. The percentage difference varied from individual to individual and also in the same individual from one day to another. Percentage differences of from 4 to 22 were found. The absolute difference was 3 mg. on an average.

In four cases the values were slightly higher for venous blood, only in a single case as much as 20 % higher.

Following the ingestion of protein, urea, diet poor in protein, fat + starch, and glucose, the main rule was again a higher concentration of urea in capillary than in venous blood in the course of the day.

Examinations of patients with diabetes, nephritis and icterus showed similar relations to those observed in normals. Only in 2 out of 22 cases was the urea content higher in venous blood.

## REFERENCES.

- Bollman, Mann and Magath (1924). *Amer. J. Physiol.* **69**, 371.  
Gad-Andresen (1921). *Biochem. Z.* **116**, 1.  
Göthgen (1925). *Hospitalstidende*, No. 14.  
Rehberg (1925, 1). *Biochem. J.* **19**, 270.  
—— (1925, 2). *Biochem. J.* **19**, 278.  
Tschudi-Madsen (1924). Studier over restkvælstoffets optræden og fordeling. (Bergen.)

# LXXV. THE INFLUENCE OF HEXOSEDIPHOSPHORIC ACID AND HEXOSEMONOPHOSPHORIC ACID UPON INSULIN HYPOGLYCAEMIA.

BY HENRY PERCY MARKS  
AND WALTER THOMAS JAMES MORGAN

*(Grocers' Company Research Scholar).*

*(From the National Institute for Medical Research, Hampstead, and  
the Biochemical Department, Lister Institute, London.)*

*(Received April 11th, 1927.)*

DURING the last twelve years a considerable amount of evidence has accumulated which points to a carbohydrate-phosphorus complex as being an essential intermediate in the normal carbohydrate metabolism. The work of Embden and his co-workers has made it appear probable that a hexosediphosphoric acid, similar to that isolated by Harden and Young [1906] from the products of yeast fermentation, is formed as an intermediate stage in the conversion of glycogen or carbohydrate to lactic acid in the muscle.

A reduction in the concentration of the inorganic phosphate in the blood following the administration of insulin has been observed by many workers, while the exact relationship between the concentrations of the inorganic phosphate and sugar in the blood has been investigated by Eadie, Macleod and Noble [1925] and by Chaikoff, Macleod, Markowitz and Simpson [1925], who found that the inorganic phosphate in the blood showed an almost parallel decrease with the blood-sugar. If, as the evidence suggests, a carbohydrate-phosphorus compound is formed as an intermediate stage in carbohydrate metabolism, the administration of this compound might result in the complete or partial amelioration of hypoglycaemic symptoms. We have therefore tried the effect of subcutaneous injections, under such conditions, of two naturally occurring phosphoric esters of this type, hexosediphosphoric ester, and hexosemonophosphoric ester.

Robison [1922] isolated from the products of yeast fermentations a hexosemonophosphoric acid which he believed was probably a mixture of glucose- and fructose-monophosphoric acids. This conclusion has recently been supported by Meyerhof [1926], who, by determining the reducing power of the substance by Willstätter and Schudel's [1918] hypiodite method, was able to distinguish between the aldose and ketose types of acid, since this method only estimates the reducing power of the aldose type. Meyerhof concluded

that Robison's acid was a mixture of about 60 % glucosemonophosphoric acid and 40 % of fructosemonophosphoric acid. Application of this method of analysis to hexosediphosphoric acid supports the conclusions of Young [1909] and Neuberg and Kretschmer [1911] that it consists almost entirely of fructose-diphosphoric acid.

#### *Preparation of the acids.*

The hexosemonophosphoric acid used was prepared from fructose by fermentation with a preparation of dried yeast. The general conditions of separation were similar to those used by Robison in his original investigation. The final barium salt, after drying over phosphorus pentoxide at 80° and 20 mm., possessed the following constants:

$$[\alpha]_{\text{H}_2\text{O}}^{18^\circ} + 16.7^\circ.$$

Phosphorus 7.45 %.

The barium salt was converted into sodium salt by shaking with the exact amount of a solution of sodium sulphate. The barium sulphate formed was removed by the centrifuge and the clear solution of sodium hexosemonophosphate was concentrated by evaporation in a vacuum desiccator.

The hexosediphosphoric acid was also prepared from fructose by fermentation with dried yeast. The final product was converted into sodium salt as described above for the monophosphate. The solution of the sodium salt was free from inorganic phosphate.

#### *Injection experiments.*

The ability of the diphosphate to relieve the symptoms of insulin hypoglycaemia was first tested on rabbits, with entirely negative results. Rabbits in insulin convulsions injected with the diphosphate showed no greater recovery than the untreated animals. Certainly there was no such dramatic recovery as is observed after the injection of a corresponding amount (1 g.) of glucose.

On account of the large quantity of material required to obtain a convincing result when using rabbits, it was decided to employ mice for further tests, since they exhibit unmistakable recovery after an injection of 100 mg. of glucose.

The mice, after receiving 0.025 unit of insulin subcutaneously, were kept in special boxes heated to 37°, since under these conditions the insulin intoxication follows a well-defined course with fatal termination, unless relieved by an injection of glucose or its equivalent. In about half an hour the mice began to show typical symptoms of insulin hypoglycaemia, namely, a condition of collapse, with legs sprawling and tails erect, or actual convulsions. Five of the affected mice received a subcutaneous injection of 0.1 g. glucose; four, an injection of an equivalent amount of hexosediphosphate; five, an injection of an equivalent amount of hexosemonophosphate; and six, the same volume of saline (0.4 cc.).

All the mice which received glucose showed definite recovery in one to two minutes and survived until the end of the experiment. Although in some cases the animals were not normal until 10 or 15 minutes later, they regained the use of their legs almost immediately.

On the other hand, those which received saline showed no amelioration of symptoms. Some of them seemed to remain alive longer than would be expected in the absence of any injection, but all were dead within  $\frac{1}{2}$  to 1 hour after the injection of saline.

The effect of both hexosediphosphate and hexosemonophosphate was in all respects similar to that of saline. One of the mice, which was the last to be injected with the diphosphate, was killed in a collapsed condition at the end of the experiment. All the others were already dead.

These experiments, therefore, afford no evidence that these two compounds can replace glucose in the relief of insulin hypoglycaemia, or that they are readily convertible into that substance in the animal body.

#### REFERENCES.

- Chaikoff, Macleod, Markowitz and Simpson (1925). *Amer. J. Physiol.* **74**, 36.  
Eadie, Macleod and Noble (1925). *Amer. J. Physiol.* **72**, 614.  
Harden and Young (1906). *Proc. Roy. Soc. Lond.* **77**, 405.  
Meyerhof (1926). *Naturwiss.* **52**, 1277.  
Neuberg and Kretschmer (1911). *Biochem. Z.* **91**, 257.  
Robison (1922). *Biochem. J.* **16**, 809.  
Willstätter and Schudel (1918). *Ber. deutsch. chem. Ges.* **51**, 780.  
Young (1909). *Proc. Roy. Soc. Lond.* **81**, 528.

## LXXVI. HAEMOCYANIN.

### PART IV. THE DEPENDENCE OF THE SHAPE OF THE OXYGEN DISSOCIATION CURVE ON THE STATE OF IONISATION OF THE PROTEIN.

BY ELLEN STEDMAN AND EDGAR STEDMAN.

*From the Department of Medical Chemistry, University of Edinburgh.*

*(Received April 16th, 1927.)*

IN the studies on the influence of  $p_H$  on the oxygen dissociation curves of the haemocyanins from *Homarus* and *Cancer* which were described in Parts II and III [Stedman and Stedman, 1926] of this investigation the shape of the dissociation curve was found to depend upon the relative amounts of pigment present as salt and in the uncombined form. The precise nature of this dependence was, however, obscure. This was largely due to the uncertainty which existed respecting the values of the isoelectric points of the pigments. The values determined by the method of optimum precipitation did not appear to correspond with the above relationship. Before further progress could be made it therefore became necessary to determine the isoelectric points by an independent method. This has now been done; the results obtained, which are in substantial agreement with those previously recorded, are presented in this communication.

This confirmation of the values obtained by the precipitation method has led to an extension of the investigation which is described below and which, the authors believe, has brought certain aspects of the influence of  $p_H$  on the dissociation curves into complete correspondence with the state of ionisation of the protein.

#### EXPERIMENTAL METHODS.

The serum employed was collected, concentrated and dialysed in the manner described in Part II; determinations of the oxygen capacity were made by the technique outlined in Part III; while  $p_H$  determinations were made electrometrically under the conditions described in Part II.

*Determination of isoelectric point by cataphoresis.* The apparatus employed for this purpose was that described by Michaelis [1925]. The dialysed serum (20 cc.) was mixed with 25 cc. of an acetic acid-sodium acetate buffer of known  $p_H$  and, in those cases where no precipitation occurred, introduced directly into the U-tube of the apparatus. When the buffer solution caused partial precipitation of the protein, the precipitate was removed by centrifuging and the supernatant liquid employed for the experiment. The large stopcocks of



the U-tube were then closed and the upper portion of the apparatus, after being thoroughly washed with distilled water, was filled with a mixture of 25 cc. of the same buffer solution with 20 cc. of water. Connection with reversible electrodes, consisting of copper wire dipping in solutions of copper sulphate, by means of small U-tubes containing potassium chloride in agar-agar was next made in the manner described by Michaelis. The subsequent operations were also made in accordance with the details given by this author. As source of E.M.F. an ordinary lighting circuit with a carbon filament lamp in series was employed. The direction of migration of the haemocyanin could be directly observed owing to the colour of this protein. The exact  $p_H$  of the protein-buffer mixture was determined electrometrically.

*Viscosity measurements.* The usual type of Ostwald viscosimeter was employed. The solutions for a given series of experiments were prepared in the following manner. A definite volume (usually 10 cc.) of the dialysed serum under examination was measured with a pipette into a graduated flask, to which were then added dilute hydrochloric acid or potassium hydroxide, in amount necessary to produce the desired change in  $p_H$ , and distilled water to the mark. Changes in  $p_H$  were, in general, effected by the addition of alkali only when the serum was on the alkaline side of the isoelectric point, and by acid only when on the acid side. The series of solutions thus prepared were allowed to stand for 15–20 hours in the ice-chest, from which they were removed individually as required for viscosimetric measurement. A definite volume (usually 8 cc.) of the haemocyanin solution was introduced into the viscosimeter which was then placed in a thermostat maintained at a temperature of 23°. The time of flow was determined after about 10 minutes.

## RESULTS.

### *Isoelectric point of haemocyanin from Cancer.*

The results of the cataphoresis experiments are shown in Table I.

Table I.

$p_H$ of serum-buffer mixture	Direction of migration	Remarks
6.20	To anode	Movement rapid
4.94	"	" slow
4.81	Mainly to anode	" very slow
4.53	To cathode	No movement to anode
4.34	"	Movement fairly rapid
4.05	"	" "

The isoelectric point of haemocyanin from *Cancer* thus clearly corresponds with a  $p_H$  value of about 4.7. The point of optimum precipitation has again been determined by the method described in Part II with the same result, namely, maximum precipitation occurred in the buffer solution at  $p_H$  4.8. On allowing the precipitate to settle, however, the supernatant liquid was found to have a  $p_H$  value of 4.88. The results of the two methods are thus in substantial agreement.

*Isoelectric point of haemocyanin from Homarus.*

The isoelectric point of haemocyanin from *Homarus* could not be determined very accurately by cataphoresis owing to the fact that it is almost completely insoluble in acetate buffers in the region of the isoelectric point. Thus complete precipitation occurred in buffers of  $p_H$  4.4, 4.6 and 4.8. The results of the cataphoresis experiments actually carried out are shown in Table II.

Table II.

$p_H$ of serum-buffer mixture	Direction of migration	Remarks
4.17	To cathode	Movement fairly rapid
4.37	Mainly to cathode	" slow
5.47	To anode	" "

The isoelectric point thus lies between the  $p_H$  values of 4.37 and 5.47. When redetermined by the method of optimum precipitation, the greatest precipitate formed in the buffer at  $p_H$  4.8. The supernatant liquid had a  $p_H$  of 4.84. It may be concluded that the isoelectric point of haemocyanin from *Homarus* is at about  $p_H$  4.7.

*Isoelectric point of haemocyanin from the snail (Helix pomatia).*

Haemocyanin from the snail differs from the pigments from *Homarus* and *Cancer* in being relatively soluble in the region of the isoelectric point. Its solubility is not sufficiently great to prevent its precipitation from dialysed serum on the addition of acid, but is large enough to render inapplicable in its simple form the method of optimum precipitation for the determination of its isoelectric point. On the other hand, this greater solubility facilitates cataphoresis experiments. Using this method, the isoelectric point of haemocyanin from snail has been found to lie at about  $p_H$  5.3, as can be seen from Table III.

Table III.

$p_H$ of serum-buffer mixture	Direction of migration	Remarks
5.56	To anode	Movement slow
5.39	Mainly to anode	" very slow
5.16	To cathode	" "

This value is confirmed by the viscosity experiments described below.

*Influence of  $p_H$  on the viscosity of solutions of haemocyanin from Cancer.*

Tables IV and V relate to experiments carried out on the alkaline and acid sides of the isoelectric point respectively.

*Influence of  $p_H$  on the viscosity of solutions of haemocyanin from Homarus.*

Measurements have only been carried out on the alkaline side of the isoelectric point. The experimental data are given in Table VI.

Table IV.

Vol. of dialysed serum (cc.)	Vol. of 0.110N KOH (cc.)	Water to (cc.)	$p_H$	Time of flow (secs.)	Relative viscosity (water = 1)
10	0	15	6.29	60.5	1.88
10	0.3	15	6.68	65.0	2.02
10	0.6	15	7.01	67.4	2.09
10	0.9	15	7.30	70.0	2.17
10	1.2	15	7.57	70.0	2.17
10	1.5	15	7.82	68.6	2.13
10	1.8	15	7.92	66.8	2.07
10	2.1	15	8.10	65.5	2.03
10	2.4	15	8.29	62.4	1.94
10	2.7	15	8.39	60.6	1.89
10	3.0	15	8.49	59.8	1.86
10	3.3	15	8.49	60.0	1.86
10	3.6	15	8.75	62.6	1.94

Time of flow for water = 32.2.

Table V.

Vol. of dialysed serum (cc.)	Vol. of 0.080N HCl (cc.)	Water to (cc.)	$p_H$	Time of flow (secs.)	Relative viscosity (water = 1)
10	0	15	4.39	48.4	1.50
10	0.3	15	4.05	48.8	1.52
10	0.6	15	4.01	49.6	1.54
10	0.9	15	3.97	51.0	1.55
10	1.2	15	4.01	52.6	1.63
10	1.5	15	4.00	54.6	1.70
10	1.8	15	3.94	57.2	1.78
10	2.1	15	3.90	60.8	1.89
10	2.4	15	3.87	65.6	2.04

Time of flow for water = 32.2.

Table VI.

Vol. of dialysed serum (cc.)	Vol. of 0.110N KOH (cc.)	Water to (cc.)	$p_H$	Time of flow (secs.)	Relative viscosity (water = 1)
10	0	15	7.59	61.2	1.90
10	0.3	15	7.95	58.7	1.82
10	0.6	15	8.22	56.7	1.76
10	0.9	15	8.42	54.8	1.70
10	1.2	15	8.69	53.6	1.66
10	1.5	15	8.84	51.6	1.60
10	1.8	15	9.04	50.6	1.57
10	2.1	15	9.26	50.2	1.56
10	2.4	15	9.58	51.4	1.60
10	2.7	15	9.88	52.4	1.63
10	3.0	15	10.07	58.2	1.81

Time of flow for water = 32.2.

*Influence of  $p_H$  on the viscosity of solutions of haemocyanin from snail (Helix pomatia).*

The difficulty experienced in dissolving precipitated haemocyanin from *Cancer* in acid commented upon in Part III was not encountered in the case of haemocyanin from snail, presumably owing to its greater solubility and possibly, also, to its greater stability in acid solution. It was thus possible

to carry out viscosity measurements on both sides of the isoelectric point with the same sample of serum. These measurements are recorded in Table VII. In the first half of the Table the  $p_H$  of the dialysed serum was changed by the addition of alkali and in the second half by the addition of acid.

Table VII.

Vol. of dialysed serum (cc.)	Vol. of 0.110N KOH or 0.098N HCl (cc.)	Water to (cc.)	$p_H$	Time of flow (secs.)	Relative viscosity (water = 1)
10	1.5 (alkali)	15	10.53	80.0	2.39
10	1.2	15	9.33	64.8	1.99
10	0.9	15	8.88	67.4	2.01
10	0.6	15	8.70	68.0	2.03
10	0.3	15	8.03	54.7	1.63
10	0	15	7.46	47.2	1.41
10	0.6 (acid)	15	4.76	41.8	1.25
10	0.9	15	4.27	47.8	1.43
10	1.2	15	3.98	55.4	1.65
10	1.5	15	3.55	64.3	1.92

Time of flow for water = 33.5.

*Determination of the  $p_H$  at which the affinity of haemocyanin from Cancer for oxygen is at a minimum.*

The variations which occur in the affinity of haemocyanin from *Cancer* for oxygen were discussed in Part III. It became necessary in connection with the viscosity experiments described above to determine as accurately as possible the  $p_H$  at which there is minimal affinity. For this purpose advantage was taken of the fact demonstrated in Part III that this particular haemocyanin is far from completely saturated with oxygen when in equilibrium with air at a temperature of 23° and at a  $p_H$  in the neighbourhood of neutrality, and a series of experiments was carried out similar to those made in Part II in another connection and illustrated in Fig. 3 of that paper. The data, which are self-explanatory, are given in Table VIII.

Table VIII.

Vol. of dialysed haemocyanin sol (cc.)	15	15	15	15	15
Vol. of 0.110N KOH (cc.)	...	0	0	0.3	1.0
Vol. of 0.008N HCl (cc.)	...	3.0	1.5	0	0
Distilled water to (cc.)	...	20	20	20	20
$p_H$	...	6.82	7.02	7.22	7.46
Oxygen content (vols. %)	...	1.336	1.256	1.212	1.262
Combined oxygen (vols. %)	...	0.747	0.667	0.623	0.673

At an oxygen pressure corresponding with that in the atmosphere (151 mm. in the experiments recorded in Table VIII) and a temperature of 23° haemocyanin from *Cancer* thus has a minimal affinity for oxygen when at a  $p_H$  of about 7.3.

It is convenient to point out here that the expression "minimal affinity" is somewhat ambiguous. It was shown in Parts II and III that certain of the oxygen dissociation curves determined at different  $[H^+]$  intersected. Obviously,

when two such curves intersect, one cannot state at which acidity the affinity of the haemocyanin is the greater unless one defines the oxygen pressure at which the affinities are being compared. In the case of the pigments from *Cancer* and *Homarus* this difficulty disappears at  $[H']$  in the neighbourhood of neutrality, as will be evident from an inspection of the curves referred to above. When in the following pages affinities at different  $[H']$  are compared without reference to the oxygen pressure under consideration, it must be understood that the comparison is limited to the initial portions of the curves, i.e. if two curves intersect the comparison is limited to oxygen pressures lower than that at which the intersection occurs.

#### DISCUSSION.

The experiments described above render necessary a re-examination of the dissociation curves determined in Parts II and III. Of these, the series of curves illustrated in Figs. 1 and 2 of Part III, which relate to the haemocyanin from *Cancer*, extend over the greater  $p_H$  range and will therefore be considered in rather more detail.

It was pointed out in Part III that there were indications that the haemocyanin employed for the curve at  $p_H$  3.84, which was the most acid solution examined, had been partly converted into a form which no longer combined reversibly with oxygen. That some drastic change in, or decomposition of, the pigment takes place at or near an acidity corresponding with this  $p_H$  receives definite confirmation from the  $p_H$ -viscosity experiments recorded above in Table V. Whilst addition of acid to the dialysed serum at  $p_H$  4.39 produced continuous increases in its viscosity, no such change occurred in the  $p_H$ . Thus, the addition of the first 0.3 cc. of acid caused the  $p_H$  to decrease to 4.05, but the further addition of 2.1 cc. scarcely produced any appreciable effect. Evidently some peculiar change occurs in the protein at a  $p_H$  of about 4.0. Changes in the external characteristics of the solution accompany this change. Thus, the initial solution ( $p_H$  4.39) employed for the experiments recorded in Table V exhibited a marked blue opalescence (Tyndall phenomenon) which diminished progressively with increasing content of acid, giving place in the mixture at  $p_H$  3.87 to a clear greenish-yellow solution. The intervening mixtures had a greenish appearance which was the more pronounced the greater the blue opalescence. One is forced to the conclusion that the loss in opalescence is indicative of a loss of power to combine reversibly with oxygen, a conclusion which is reinforced by the observation, made during the  $p_H$  determinations, that the opalescence disappears entirely when hydrogen is bubbled through the solutions whereas the greenish-yellow colour is not affected by this treatment. Since, further, the loss of opalescence following the addition of acid takes place slowly, it is clear that the dissociation curve at  $p_H$  3.84 may be subject to errors arising from this cause. Such a possibility was tacitly suggested in Part III, and in view of the above results the position of the curve must be regarded as doubtful. There then remain in the immediate

neighbourhood of the isoelectric point, which has now been definitely shown to lie at about  $p_H$  4.7 (see Table I), two curves, one on the acid side ( $p_H$  4.19) and one on the alkaline side ( $p_H$  5.60). The former is steeper than the latter, but in view of the fact that the  $p_H$  interval between it and the isoelectric point is the smaller, this is not inconsistent with the occurrence of a maximal affinity of the pigment for oxygen at the isoelectric point. Unfortunately it is doubtful, owing to the insolubility of the pigment in the zone surrounding the isoelectric point and to the above-mentioned change which occurs at  $p_H$  4, whether the existence of such a maximal affinity could be directly demonstrated. Nevertheless, the probability that it does exist seems very great, although the occurrence of a maximal affinity on the acid side of, and not at, the isoelectric point would not be inconsistent with the results discussed below.

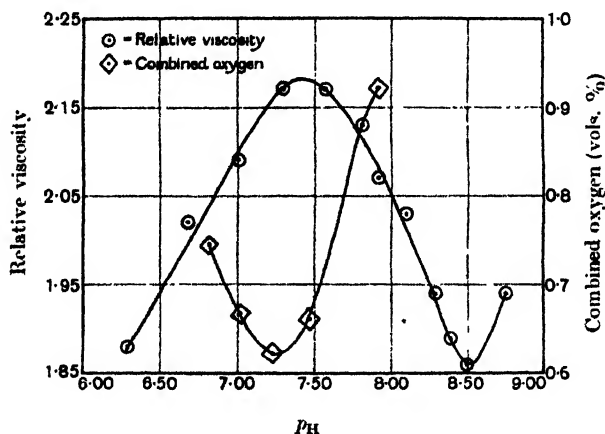


Fig. 1.

The variations in affinity which occur on the alkaline side of the isoelectric point must now be considered in connection with the viscosity determinations. For this purpose the results given in Tables IV and VIII have been plotted in Fig. 1. It can be seen that not only does the decrease in affinity which takes place as one recedes from the isoelectric point correspond, as was to be expected, with an increase in viscosity, but, what is more significant, the  $p_H$  (7.3) at which the affinity is at a minimum coincides with that at which the viscosity is at a maximum. Further, if one examines the dissociation curves illustrated in Part III it is evident that the curves (Fig. 2) determined at  $p_H$ 's more acid than 7.3 are not the exact counterpart of those (Fig. 1) determined at more alkaline  $p_H$ 's. On the contrary, while the latter are definitely of the familiar S-shape, the former do not show a marked point of inflexion. In fact, the first few points of the curves at  $p_H$  4.19, 5.60 and 6.21 lie on straight lines passing through the origin. Whilst the possibility of a slight inflexion in the curves, such as that actually illustrated, cannot be definitely excluded it would seem that between the isoelectric point and the point of maximal

viscosity the amount of oxygen taken up in combination is, within the limits of experimental error, initially proportional to the oxygen pressure.

The viscosity experiments (Table VI) on haemocyanin from *Homarus*, the results of which are plotted in Fig. 2, are in conformity with the above relationship. Owing, however, to the more extensive zone of insolubility exhibited by this pigment in the region of the isoelectric point the authors have not hitherto succeeded in demonstrating the occurrence of either a point of maximal viscosity or one of minimal affinity. Nevertheless, it is clear from Fig. 1 in Part II that if a point of minimal affinity exist it will occur at a  $p_H$  of less than 7.76. By analogy with other proteins it is also evident from Fig. 2 that a point of maximal viscosity will likewise occur at a  $p_H$  somewhat lower than 7.59. In view of the results obtained with haemocyanin from *Cancer* there seems little doubt that these two points will coincide. One further point merits mention in connection with *Homarus*. It will be recalled that at  $p_H$  9.65 the combination with oxygen of the pigment from this species follows closely, if

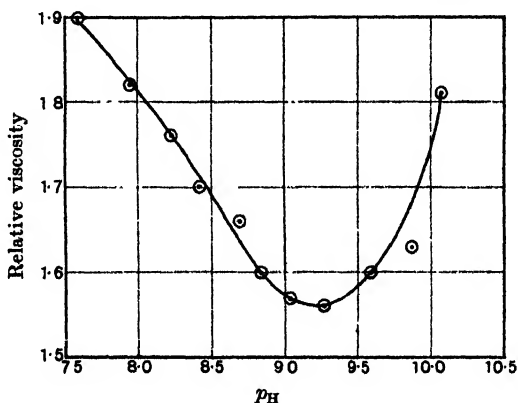


Fig. 2.

not completely, the laws applicable to homogeneous solutions. Reference to Fig. 2 shows that at this  $p_H$  the viscosity lies on the alkaline side of a minimum in the  $p_H$ -viscosity curve. The increase in viscosity which is taking place evidently represents the commencement of those processes which finally produce, in more alkaline solutions, gelation.

Although the determinations of the viscosity of solutions of haemocyanin from snail (Table VII) were made primarily with the object of confirming the position of the isoelectric point, an inspection of Fig. 3, in which the results are plotted, shows that a maximal viscosity occurs on the alkaline side of the isoelectric point at a  $p_H$  of about 8.5. It is thus possible to predict that the affinity of this haemocyanin will be at a minimum in the neighbourhood of this  $p_H$ .

The examination of further respiratory pigments will doubtless reveal relationships between viscosity and affinity similar to that demonstrated in this communication. Whilst it is to be expected that the relationship will be

of the same precise nature, it can be foreseen that it will be less marked with certain pigments. Loeb [1922] has shown that the viscosity of solutions of proteins, such as egg-albumin, which are readily crystallisable, is influenced by changes in  $p_H$  to a smaller degree than, for example, that of a gelatin solution. Whilst, as shown by Pauli and his co-workers [1925, 1926], changes of the same nature do actually occur with both types of protein, it is evident from Loeb's work that they are less pronounced in the case of the crystallisable proteins. In other words, the more readily a protein can be crystallised the less marked are its colloidal properties. The haemocyanins from *Cancer* and *Homarus* are examples of proteins with pronounced colloidal properties. As a result, the influence of  $p_H$  on both the viscosity and the power of combination with oxygen of these proteins is very great. The haemoglobins are less colloidal in nature; hence the relationship between viscosity and oxygen-combining power will not be so evident although it should be no less precise than in the case of the haemocyanins.

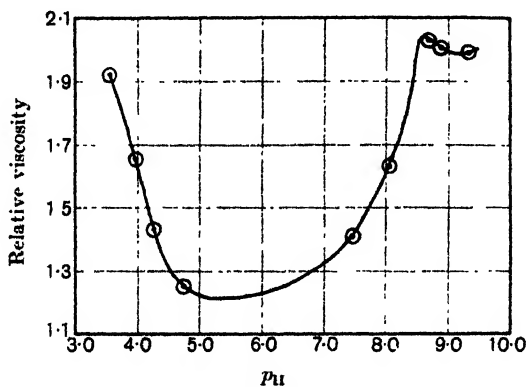


Fig. 3.

The ultimate bearing of the relationship discussed above on the mechanism of the combination between oxygen and respiratory pigments cannot yet be foreseen, but certain factors appear to be quite evident. Maximal affinity for oxygen probably corresponds with minimal ionisation and minimal viscosity, *i.e.* occurs at the isoelectric point; the alternative view that the point of maximal affinity occurs on the acid side of the isoelectric point and corresponds with maximal ionisation of the protein as cation cannot, however, be entirely excluded. As stated above, it is unfortunately scarcely possible to distinguish between these two possibilities in the case of haemocyanin from *Cancer* owing to the limits of stability of this pigment. According to Pauli [1926], maximal viscosity in proteins indicates maximal ionisation; hence, since in the case of certain haemocyanins the former corresponds with minimal affinity, it would appear that the greater the degree of ionisation (as anion) of the pigment the smaller is its affinity for oxygen. As the ionisation is repressed by increased alkalinity of the solution the affinity again increases. Finally, at  $[H^+]$  at which



the hydration processes which ultimately result in gelation have commenced the combination with oxygen takes place according to the laws for true solutions. So much seems clear from the combined experiments on *Cancer* and *Homarus* haemocyanins. Why the affinity should vary with the ionisation is not so clear, nor is it apparent why the curves on the acid and alkaline sides of the point of maximal viscosity should be of such a markedly divergent character. One factor which must certainly be taken into account in this connection is the hydration of the micellae. According to Pauli's views the point of maximal viscosity corresponds with maximal hydration of the micellae, due to the greater affinity of the ions for water, from which one might infer that the affinity for oxygen is influenced primarily by hydration and is only affected by ionisation in so far as this influences the hydration of the micellae. But the question arises: is the viscosity curve an accurate indicator of the state of hydration of the micellae? According to Smoluchowski [see Freundlich, 1926] the presence of a charge on the micellae of a sol produces an increase in the viscosity of the sol. Since maximal viscosity in a protein sol corresponds with maximal ionisation, i.e. with maximal charge on the micellae, it is conceivable, indeed probable, that the variation in viscosity is conditioned largely, if not mainly, by the variation in the charge on the micellae and not by variation in the state of hydration. On this basis it would be necessary to assume that the hydration of the micellae increases uniformly, or at any rate without passing through a maximum, as one recedes from the isoelectric point until finally the sol undergoes gelation. The affinity would then be directly controlled in, it must be admitted, an unknown manner by the magnitude and perhaps also the sign of the charge on the micellae, whilst the actual contour of the curve would be determined by the degree of hydration of the micellae. (The sign of the charge could only be considered of influence if it were shown that maximal affinity occurred on the acid side of the isoelectric point and corresponded with maximal ionisation of the protein as cation; such a result would be of great theoretical interest, but its consideration must be postponed until it has been found possible to distinguish experimentally between the two possibilities.) This would account for the different shapes of the curves on the two sides of the point of maximal viscosity. It would further account for the observation that with increasing alkalinity the curve approximates more and more closely to the hyperbolic type, for it is recognised that a protein gel resembles in many of its properties a true solution much more nearly than does a protein sol; this is presumably due to the facts that the micellae are highly hydrated and that the volume occupied by the intermicellar liquid is negligible in comparison with that occupied by the micellae.

#### SUMMARY.

The isoelectric points of the haemocyanins from *Cancer* and *Homarus* are at  $p_H$  4.7; that of haemocyanin from the snail (*Helix pomatia*) is at  $p_H$  5.3.

The viscosity of solutions of haemocyanin from *Cancer* passes through a

maximum at  $p_H$  7.3; at the same  $p_H$  the affinity of the pigment for oxygen passes through a minimum. Viscosity determinations with solutions of haemocyanin from *Homarus* suggest that a similar relationship holds in the case of this pigment.

The above results taken in conjunction with the shape of the oxygen dissociation curves determined at different  $p_H$  lead to the following conclusions. The affinity of the pigment for oxygen is probably at a maximum at the isoelectric point; alternatively, maximal affinity occurs on the acid side of the isoelectric point at a  $p_H$  corresponding with maximal ionisation of the protein as cation. It is not at present possible to differentiate between these two possibilities owing to an abnormal change which haemocyanin from *Cancer* undergoes at  $p_H$  4. As the pigment ionises as anion its affinity for oxygen decreases, passes through a minimum at a  $p_H$  at which ionisation is at a maximum, and increases again as the ionisation is repressed by the presence of higher concentrations of alkali. Finally, as the  $p_H$  approaches more and more closely to that at which gelation occurs, the dissociation curve approximates more and more closely to the type to be expected if the combination obeyed the law of mass action. The shape of the curve is controlled by two factors: the charge on the micellae, and their state of hydration.

The expenses of this investigation, which has been carried out during the tenure, by one of us, of a Carnegie Teaching Fellowship, have been met by grants from the Government Grant Committee of the Royal Society and the Moray Research Fund of this University.

#### REFERENCES.

- Freundlich (1926). *Colloid and Capillary Chemistry*, 369.  
Frisch, Pauli and Valkó (1925). *Biochem. Z.* **164**, 401.  
Loeb (1922). *Proteins and the Theory of Colloidal Behaviour*, 198.  
Michaelis (1925). *Practical Physical and Colloid Chemistry*, 121.  
Pauli (1926). *Eiweisskörper und Kolloide*.  
Pauli and Wit (1926). *Biochem. Z.* **174**, 308.  
Stedman and Stedman (1926). *Biochem. J.* **20**, 938, 949.

# LXXVII. THE EFFECT OF HALOGEN SALTS ON PEPTIC DIGESTION.

BY WINIFRED MARY CLIFFORD.

*From the Physiological Laboratory, Household and Social Science Department,  
King's College for Women, Kensington, London, W. 8.*

*(Received April 20th, 1927.)*

IN a previous paper [Clifford, 1925] the effects of the halogen salts of sodium, potassium, ammonium, and calcium on salivary digestion were described. These salts were found to vary in their action on ptyalin, some delaying digestion, others being inert, and some increasing the speed of reaching the achromic point.

It was thought advisable to continue the investigation with other enzymes, and the experiments to be described give results obtained as to the effects of halogen salts on the action of pepsin. Very little work appears to have been done on the influence of salts on peptic digestion. Hamburger and Halpern [1916] found that sodium chloride in "high concentration" only (0.25 %) inhibited peptic activity, whilst in "low concentration" (0.170 %) it accelerated it. They obtained similar results with sodium phosphate.

Rona and Kleinmann [1924] investigated the effects of the chlorates, nitrates and sulphates of various metals, and using a nephelometric method found that all the salts hastened peptic digestion if the  $p_H$  of the solution were high and the concentration of the salts low, whilst the reverse was the case if the  $p_H$  were low or the salts more concentrated. Cole [1926, p. 248] says "neutral salts inhibit the action of pepsin, this being in marked contrast to the influence of sodium chloride on ptyalin." Bayliss [1924] states, as a generalisation on ferments, that "neutral salts also have an influence, as a rule, of a favourable kind."

## EXPERIMENTAL METHOD.

The end-point, or any specific intermediate point of ordinary peptic digestion is difficult to ascertain, since there is no ready method of estimating the amount of peptone and polypeptides produced. Precipitation methods, such as Fuld's described by Cole [1926, p. 252], could not be used in experiments involving the use of halogen salts, for such salts would precipitate part of the edestin and obscure the end-point of the experiment. Finally it was decided to use the clotting of milk, recommended by Cole [1926, p. 249], as a measure of peptic activity, since it is a definite and easily seen change.

The actual experimental method was as follows.

10 cc. milk were pipetted into each of several test-tubes, standard solutions of the halogen salts in other tubes, and for control experiments distilled water in another tube. These solutions were brought to 37° in a water-bath. Then to one of the milk tubes 2 cc. of the distilled water or salt solution were added, and 2 cc. of the pepsin solution. The tube was returned to the bath, and a stop-watch started. At the moment when clotting began the watch was stopped. In this way a definite end-point could be seen and the effect of the salts on the rate of coagulation noted. The strength of the pepsin solution was adjusted till 10 cc. milk, 2 cc. water, and 2 cc. pepsin clotted in 1½ to 3 minutes.

To determine the moment of clotting, the test-tube was removed from the water-bath, tilted gently and replaced as rapidly as possible. This produced a thin film of milk which trickled down the side of the tube. The tilting was repeated every 30 seconds. When clotting was incipient the film became flocculent instead of homogeneous, and the whole tube of milk became solid about 2-5 seconds after the formation of the clotted film.

After the first tube had been roughly tested in this manner, the next tube was left in the bath till 15 seconds before the anticipated time of coagulation. It was then tilted as described, returned to the bath, and generally the film had not time to fall before becoming flocculent. If this were not the case, the tube was again tilted, and the start of coagulation looked for in the new film. In this manner cooling with consequent retardation of enzyme action was minimised.

The figures given in the following tables for each strength of each salt represent the average of results obtained from six tubes. The greatest difference in any one set of six was 8 seconds.

Actual figures for one of the standard experiments with 2 cc. water are 112, 108, 108, 110, 111, 112 seconds.

Two separate batches of commercial pepsin scales were used in these experiments, one requiring a 0.25 % solution, and the other a 0.4 %. At least two separate samples of each halogen salt were also tested.

The concentration of halogen salts varied from 2 cc. of 2*M* solution to 2 cc. of 0.062 *M* solution, each successive dilution being 50 % of the previous one.

Since these 2 cc. were in a bulk of 14 cc. liquid, the actual experimental concentration was  $\frac{1}{7}$  of the weighed halogen solution.

## EXPERIMENTAL RESULTS.

### Fluorides.

[illegible]

From these results it is seen that these soluble fluorides completely inhibit the clotting of milk by pepsin in concentrations greater than 0.035 *M* for the Na salt and 0.0175 *M* for the K and NH<sub>4</sub> salts. In the lower concentrations the action is reversed and the rate of coagulation hastened by the fluorides.

### Chlorides.

Conc. <i>M</i>	LiCl	NaCl	KCl	NH <sub>4</sub> Cl
0.28	1 min. 7 secs.	1 min. 22 secs.	1 min. 50 secs.	0 min. 40 secs.
0.14	1 " 0 "	0 " 57 "	1 " 27 "	0 " 34 "
0.07	0 " 53 "	0 " 49 "	1 " 0 "	0 " 30 "
0.035	0 " 56 "	0 " 50 "	1 " 2 "	0 " 32 "
0.0175	1 " 3 "	1 " 0 "	1 " 2 "	0 " 32 "
0.00875	1 " 12 "	—	1 " 53 "	1 " 30 "
0.0 (control)	1 " 45 "	1 min. 45 secs.	2 " 6 "	2 " 6 "

These figures show that the chlorides hasten the rate of clotting, the ammonium salt being the most active.

There is also an optimum concentration for this reaction ranging between 0.07 molar and 0.018 molar, above and below which the acceleration is less marked.

As far as this phase of digestion is concerned, there is certainly no inhibitory effect exerted by NaCl on pepsin in a concentration of approximately 1.5 %, though Hamburger and Halpern [1916] state that a concentration of NaCl above 0.25 % inhibits peptic activity.

Conc. <i>M</i>	MgCl <sub>2</sub>	CaCl <sub>2</sub>	BaCl <sub>2</sub>
0.28	0 min. 41 secs.	Instantaneous	Instantaneous
0.14	0 " 4 "	"	"
0.07	Instantaneous	"	"
0.035	"	"	"
0.0175	"	"	"
0.00875	"	"	"
0.0 (control)	2 mins. 40 secs.	1 min. 47 secs.	3 mins. 0 secs.

The salts of this group, as expected from the well-known action of CaCl<sub>2</sub> on clotting reactions, produced a great increase in the rate of coagulation. This increase was much less marked with MgCl<sub>2</sub> in the higher concentrations, though in smaller concentration it equalled that given by the Ca and Ba salts.

### Bromides.

The results obtained with the bromides are given in the curves in Fig. 1. They are similar to those obtained with chlorides.

The existence of an optimum concentration, however, is more strongly marked (Fig. 1) where it can be seen that with NaBr and KBr a concentration of 0.28 *M* causes definite retarding action. With KBr the hastening is only feebly shown with a concentration as low as 0.0175 *M*. Again the NH<sub>4</sub> salt is the most active.

Conc. <i>M</i>	MgBr <sub>2</sub>	CaBr <sub>2</sub>	BaBr <sub>2</sub>
0.28	1 min. 17 secs.	Instantaneous	Instantaneous
0.14	0 " 9 "	"	"
0.07	0 " 4 "	"	"
0.035	0 " 3 "	"	"
0.0175	0 " 3 "	"	"
0.00875	"	"	"
0.0 (control)	1 min. 54 secs.	1 min. 54 secs.	1 min. 54 secs.

These results are similar to those with the corresponding chlorides, viz. a marked hastening which is more marked with Ca and Ba than with Mg. Again the higher concentrations of the Mg salt are less powerful than the lower.

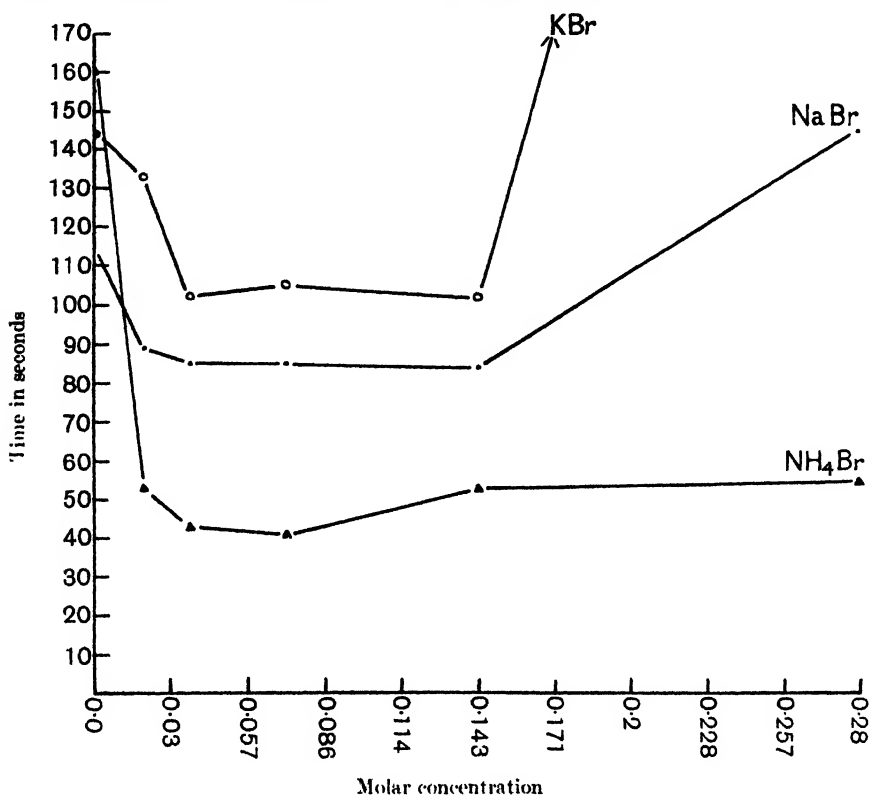


Fig. 1.

### Iodides.

Conc. <i>M</i>	LiI	NaI	KI	NH <sub>4</sub> I
0.28	3 hrs. 0 mins. } No	2 hrs. 20 mins. } No	1 hr. 54 mins. } No	5 mins. 7 secs.
0.14	3 " 0 " } clot	2 " 20 " } clot	1 " 54 " } clot	3 " 8 "
0.07	3 " 0 " }	2 " 20 " }	1 " 54 " }	2 " 34 "
0.035	41 mins.*	3 mins. 36 secs.	3 mins. 18 secs.	1 " 26 "
0.0175	2 mins. 15 secs.	1 " 54 "	1 " 42 "	1 " 29 "
0.00875	1 " 43 "	-	-	-
0.0 (control)	1 " 43 "	1 min. 54 secs.	1 min. 54 secs.	2 mins. 55 secs.

\* This clot was very fine in each of the six tubes. The milk never became solid, but showed a distinctly granular appearance.

This series shows marked differences. The iodides of Li, Na and K completely stop the coagulative action of pepsin on milk up to a concentration of 0.07 *M*, whilst the NH<sub>4</sub> salt causes a comparatively slight retardation up to a concentration of 0.14 *M*, and then accelerates the rate.

The Li, Na, and K salts in low concentrations appear to have no effect on the rate of coagulation of milk by pepsin.

Conc. <i>M</i>	MgI <sub>2</sub>	CaI <sub>2</sub>	BaI <sub>2</sub>
0.28	0 mins. 51 secs.	Instantaneous	Instantaneous
0.14	0 " 14 "	"	"
0.07	0 " 9 "	"	"
0.035	0 " 10 "	"	"
0.0175	0 " 11 "	"	"
0.00875	—	"	"
0.0 (control)	3 mins. 0 secs.	3 mins. 0 secs.	3 mins. 0 secs.

This series is similar to the other two alkaline earth series. All show a marked hastening influence on coagulation which is much less pronounced with the Mg salt than with the salts of Ca and Ba.

From the results with the Na, K and NH<sub>4</sub> series, it seems that the halide radicle is the primary factor in altering the coagulation rate. The ammonium radicle, however, influences the reaction in a favourable manner.

With the Mg, Ca, Ba series the quickening influence is a function of the metallic radicle, Mg being by far the least potent.

These results are dependent on concentration, for in any series a high concentration of salt was accompanied by an increase in the time for coagulation to occur, except with salts of Ca and Ba where the influence of the metallic radicle is overpowering in any of the strengths used.

#### SUMMARY.

(1) The clotting of milk by pepsin is greatly affected by the addition of halogen salts.

(2) The reaction is hastened by the chlorides and bromides of Na, K and NH<sub>4</sub>, the NH<sub>4</sub> salts having the strongest effect.

(3) There is an optimum concentration of Na, K and NH<sub>4</sub> chlorides and bromides which lies between 0.07 and 0.018 *M*.

(4) The iodides and fluorides of Na, K and NH<sub>4</sub> on the whole retard or inhibit the action of pepsin in coagulating milk.

(5) The inhibitory action of NH<sub>4</sub>I is very weak compared with that of NaI and KI.

(6) This retarding action is also a function of concentration, since at low concentrations the iodides do not affect the rate, whilst the fluorides actually hasten it.

(7) The NH<sub>4</sub> radicle favours the coagulation of milk by pepsin.

(8) In the presence of Mg, Ca and Ba halides there is a great increase in the rate of coagulation of milk by pepsin. This action is weakest with the Mg salts.

The expenses of this research were defrayed by a grant from the Medical Research Council.

#### REFERENCES.

- Bayliss (1924). *Principles of General Physiology*, 331.  
 Clifford (1925). *Biochem. J.* **19**, 218.  
 Cole (1926). *Practical Physiological Chemistry*.  
 Hamburger and Halpern (1916). *Arch. Int. Med.* *Chicago*, **18**, 228  
 Rona and Kleinmann (1924). *Biochem. Z.* **150**, 444.

## LXXVIII. STUDIES IN CARBOHYDRATE METABOLISM.

### III. THE INFLUENCE OF DIHYDROXYACETONE UPON THE RESPIRATORY METABOLISM AND UPON THE INORGANIC PHOSPHATE OF THE BLOOD.

BY CHARLES GEORGE LAMBIE  
AND FRANCES AGNES REDHEAD.

*From the Department of Therapeutics, Edinburgh University, and  
the Research Laboratory of the Royal College of Physicians, Edinburgh.*

*(Received April 26th, 1927.)*

Two divergent views are at present held regarding the path by which dihydroxyacetone is metabolised. According to one view—that of Campbell, Fletcher, Hepburn and Markowitz [1926]—it is first of all entirely converted into glucose which is then metabolised in the ordinary way.

According to the second view, dihydroxyacetone may be in part directly oxidised, although, in the diabetic, it may be more or less completely converted into glucose according to the severity of the condition, whilst even in the normal individual a certain amount may go into glucose, particularly after large doses [Kermack, Lambie and Slater, 1927]. Indirect evidence pointing to direct utilisation is afforded by the observation that the blood-sugar curves, both in diabetic and in normal subjects, after administration of dihydroxyacetone (50 g.) not only do not rise to so great a height as after an equivalent amount of glucose, but return more rapidly to the original level [Isaac and Adler, 1924; Rabinowitch, 1925; Kermack, Lambie and Slater, 1926; Mason, 1926, 1]. As regards recovery from insulin hypoglycaemia, Kermack, Lambie and Slater [1927] could find no significant difference between the time taken to remove the symptoms after administration of the same dose of glucose and of dihydroxyacetone. Moreover, after dihydroxyacetone recovery sometimes occurred in spite of the fact that the blood-sugar had apparently not risen to the "convulsion level." More direct evidence, suggestive of direct utilisation, was furnished by the experiment upon the decerebrated cat with the liver excluded from the circulation, in which it was shown that the muscles removed dihydroxyacetone from the blood much more rapidly than glucose when the two substances were transfused at an equal rate. This experiment does not, however, tell us whether the dihydroxyacetone which is removed by the muscles is oxidised or whether it is merely stored in some form.

In order to settle the question of direct oxidation it would be necessary to show that dihydroxyacetone could raise both the respiratory quotient and



the oxygen consumption more rapidly than glucose. If it could be demonstrated that these changes in the respiratory metabolism occur in the diabetic after administration of dihydroxyacetone under conditions where they are absent or less marked after glucose, the evidence in favour of direct oxidation would be further strengthened.

Mason [1926, 2] has found that dihydroxyacetone when administered by the mouth does, as a matter of fact, raise the respiratory quotient and the metabolic rate more rapidly and more markedly than an equal amount of glucose, and this occurred both in diabetic and in normal individuals. A possible objection, may, however, be raised to the conclusions drawn from his experiments owing to the consideration that the differences observed may have been due to dihydroxyacetone being more rapidly absorbed from the alimentary canal than glucose. It was therefore decided to repeat Mason's observations and to compare the effects of dihydroxyacetone and glucose upon the metabolism after intravenous administration. A few similar experiments were also performed with laevulose. In order to see whether the observations of Campbell, Fletcher, Hepburn and Markowitz regarding the identical behaviour of the inorganic blood-phosphate curves after administration of glucose and dihydroxyacetone could be confirmed, these were followed simultaneously with those of the respiratory metabolism and the blood-sugar.

#### EXPERIMENTAL.

In all experiments the subject was in the post-absorptive condition. The expired air was collected in 6-minute samples, using the Douglas bag method, and was analysed by means of Haldane's apparatus. The basal metabolic rate was first determined and a sample of blood for blood-sugar, blood-dihydroxyacetone and blood-inorganic phosphate was taken. The blood-sugar was determined by MacLean's method, the inorganic phosphate by Briggs' [1922] modification of the Bell and Doisy method and dihydroxyacetone by Campbell and co-workers' [1926] method. After administration of the carbohydrate, similar determinations were made at intervals.

In the first series of experiments oral administration was employed.

#### *Oral administration.*

*Exp. 1 (a).* Respiratory metabolism and curves of blood-sugar and blood-phosphate in normal student after ingestion of 50 g. glucose.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood- sugar mg. %	Blood- inorganic phosphate mg. %
0	- 3	1.484	0.81	31.5	94	3.629
Glucose 50 g.						
18	± 0	1.486	0.91	32.1	111	3.629
31	+ 10	1.712	0.75	35.0	109	3.605
46	+ 10	1.700	0.76	34.0	100	3.358
61	+ 4	1.663	0.79	34.5	87	3.388
91	+ 4	1.695	0.84	33.25	84	3.750
128	+ 1	1.564	0.76	32.0	75	3.308
173	- 13	1.378	0.8	27.85	87	3.358

*Exp. 1 (b).* Same as preceding after ingestion of 50 g. dihydroxyacetone.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-dihydr. mg. %	Blood-inorganic phosphate mg. %
0	+ 3	1.643	0.75	28.32	86	0	3.432
Dihydroxyacetone 50 g.							
15	+ 21	1.779	1.10	39.36	86	5	2.848
20	+ 35	1.976	1.10	46.50	105	30	2.526
44	+ 31	1.941	1.10	48.90	106	49	2.368
67	+ 10	1.614	1.10	45.21	87	25	2.586
97	+ 4	1.469	1.10	40.56	68	5	2.884
127	+ 13*	1.909	0.707	35.10	76	5	2.960
157	- 3	1.593	0.67	35.10	68	0	2.960

\* Mask readjusted.

*Exp. 2 (a).* Respiratory metabolism and curves of blood-sugar and blood-phosphate in normal student after ingestion of 50 g. glucose.

Time mins.	Metabolic rate % of normal B.M.R.	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-inorganic phosphate mg. %
0	- 1	1.750	0.81	37.2	117	3.243
Glucose 50 g.						
19	+ 2	1.789	0.72	36.0	123	3.236
38	+ 18	2.092	0.76	41.6	113	3.000
47	+ 15	1.957	0.85	43.5	110	2.925
59	+ 19	2.091	0.73	42.5	76	2.703
88	+ 13	2.005	0.7	40.2	67	2.928
116	+ 7	1.865	0.76	37.6	80	2.756

*Exp. 2 (b).* Same as preceding after 50 g. laevulose.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-laevulose mg. %	Blood-inorganic phosphate mg. %
0	+ 15	1.620	0.8	40.5	110	0	2.008
Laevulose 50 g.							
25	+ 26	1.817	0.72	40.0	98	25	1.825
39	+ 20	1.716	0.75	39.0	69	0	1.759
49	+ 1	1.409	0.84	35.5	-	-	-
68	+ 8	1.460	0.82	35.0	-	-	-
95	+ 10	1.555	0.85	36.0	70	0	1.715

*Exp. 3 (a).* Respiratory metabolism and curves of blood-sugar and blood-phosphate in a diabetic patient after ingestion of 50 g. glucose.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-inorganic phosphate mg. %
0	+ 18	1.339	0.60	26.35	168	3.571
Glucose 50 g.						
15	+ 7	1.051	0.66	23.10	192	3.562
34	- 3	1.149	0.62	23.50	204	3.556
49	+ 15	1.317	0.59	24.25	251	3.571
69	+ 11	1.259	0.63	24.25	295	2.777
109	+ 7	1.200	0.65	24.25	341	2.900
149	+ 5	1.192	0.63	23.75	312	3.409
190	- 5	1.091	0.56	24.35	255	3.333

*Exp. 3 (b).* Same as preceding after ingestion of 50 g. dihydroxyacetone.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-dihydr. mg. %	Blood-inorganic phosphate mg. %
0	+ 2	1.147	0.65	24.50	187	0	3.1846
Dihydroxyacetone 50 g.							
24	+ 9	1.173	0.85	26.65	206	60	2.568
46	+ 50	1.615	0.80	37.39	230	70	2.272
65	+ 29	1.423	0.71	32.50	270	55	2.343
98	+ 14	1.285	0.66	28.43	249	25	2.922
132	+ 2	1.150	0.65	23.00	231	10	2.445
160	+ 5	1.182	0.63	23.61	243	5	2.445

*Exp. 3 (c).* Same as preceding after ingestion of 50 g. laevulose.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-inorganic phosphate mg. %
0	± 0	1.227	0.57	24.15	133	3.703
Laevulose 50 g.						
15	- 7	1.234	0.63	24.25	173	3.703
30	+ 16	1.370	0.69	27.5	202	3.750
46	+ 15	1.367	0.68	28.0	222	3.571
60	+ 18	1.390	0.72	29.75	222	3.845
90	+ 5	1.227	0.68	25.0	222	?
123	+ 2	1.140	0.99	25.0	202	3.488

*Exp. 4 (a).* Respiratory metabolism and curves of blood-sugar and blood-phosphate in a diabetic patient after ingestion of 50 g. glucose.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-inorganic phosphate mg. %
0	- 15	1.063	0.79	24.90	152	3.304
Glucose 50 g.						
51	± 0	1.360	0.6	27.34	253	3.300
82	+ 6	1.371	0.62	28.50	400	2.905
143	23	0.801	0.51	22.50	444	3.321

*Exp. 4 (b).* Same as preceding after ingestion of 50 g. dihydroxyacetone.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-dihydr. mg. %	Blood-inorganic phosphate mg. %
0	- 12	1.134	0.65	30.50	145	5	3.289
Dihydroxyacetone 50 g.							
12	11	1.116	0.74	30.50	190	25	2.952
33	+ 44	1.818	0.74	46.25	202	55	2.686
53	+ 37	1.819	0.73	48.25	253	35	2.343
73	- 10	1.159	0.66	28.20	—	—	—
140	- 10	1.159	0.65	28.75	222	5	2.393
179	- 20	1.047	0.61	27.50	210	5	2.678

*Intravenous administration.*

In the next series the substances were injected intravenously.

The solutions for intravenous injection were made by dissolving 25 g. glucose or dihydroxyacetone in physiological saline in the cold and making up the volume to 50 cc. Sterilisation of the solution was effected by passing it through a Seitz germicide (EK) filter No. 6, using special pads impermeable to micro-organisms. 40 cc. of the fluid, previously warmed to 37°, were injected into a superficial vein at the bend of the elbow, the injection being made at a uniform rate and occupying a period of exactly 5 minutes. Strong solutions were employed in order to avoid excessive blood-dilution, and to minimise any disturbing effects upon metabolism occasioned by the injection of large volumes of fluid. Great care must, however, be exercised in the use of such hypertonic solutions owing to the risk of producing venous thrombosis. While the injection was in progress there occurred, with dihydroxyacetone, an obvious increase in the depth and frequency of the respiratory movements. At first it was thought that this might have been due to the direct stimulation of the respiratory centre by dihydroxyacetone, or to something of the nature of a "specific dynamic action." The analyses of 3-minute samples of expired air taken during this period show, however, that the increased breathing was accompanied by a rise in the total metabolism, in the oxygen consumption and in the R.Q., indicating that the carbohydrate itself was being rapidly metabolised. These phenomena were not observed in the case of glucose.

*Exp. 5 (a).* Respiratory metabolism and curves of blood-sugar and blood-phosphate in a normal student after intravenous injection of 20 g. glucose.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-inorganic phosphate mg. %
0	+ 7	1.766	0.73	37.5	85	3.515
1.5 Glucose 20 g.						
16	+ 8	1.760	0.79	43.35	156	3.260
31	+ 24	2.441	0.80	43.75	112	3.571
44	+ 19	1.887	0.82	42.5	75	4.40
58	+ 17	1.904	0.70	40.0	67	3.571
74	+ 16	1.927	0.78	41.8	67	3.375
103	+ 15	1.898	0.73	41.35	76	4.40
136	+ 8	1.774	0.72	37.20	73	—

*Exp. 5 (b).* Same as preceding after intravenous injection of 20 g. dihydroxyacetone.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-dihydr. mg. %	Blood-inorganic phosphate mg. %
0	- 7	1.59	0.83	39.35	90	15	3.125
1.5 Dihydroxyacetone 20 g.							
1.3	+ 15	1.94	0.87	42.00	—	—	—
3.6	+ 50	2.51	0.92	61.50	—	—	—
17	+ 49	2.54	0.86	57.00	92	20	2.240
27	+ 36	2.35	0.81	51.00	93	15	2.312
40	+ 12	1.91	0.84	47.00	82	15	2.351
52	+ 7	1.85	0.80	46.50	70	15	2.700
67	+ 3	1.8	0.76	40.25	69	15	2.725

*Exp. 6 (a).* Respiratory metabolism and curves of blood-sugar and blood-phosphate in a diabetic patient after intravenous injection of 20 g. glucose.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-inorganic phosphate mg. %
0	+ 1	1.239	0.5	25.5	168	3.750
1-5 Glucose 20 g.						
12	- 1	1.188	0.5	25.0	245	3.685
25	+ 8	1.381	0.51	27.0	236	3.597
40	+ 10	1.274	0.62	28.0	205	3.456
60	- 4	1.220	0.56	27.0	212	3.658
68	- 4	1.125	0.56	25.0	195	3.658
105	- 4	1.115	0.52	25.0	167	3.428

*Exp. 6 (b).* Same as preceding after intravenous injection of 20 g. dihydroxyacetone.

Time mins.	Metabolic rate	Oxygen consumption l. per 6 mins.	R.Q.	Expired air l. per 6 mins.	Blood-sugar mg. %	Blood-dihydr. mg. %	Blood-inorganic phosphate mg. %
0	+ 2	1.227	0.55	27.50	202	80.0	3.333
1-5 Dihydroxyacetone 20 g.							
1-3	+ 46	1.735	0.59	35.20	—	—	—
3-6	+ 59	1.792	0.85	41.00	—	—	—
16	+ 56	1.749	0.88	41.75	253	35.0	2.884
33	+ 21	1.687	0.88	34.00	246	27.5	2.812
47	- 10	1.030	0.73	29.00	246	20.0	2.585
63	- 6	1.071	0.79	31.00	222	15.0	2.585
95	+ 0	1.201	0.60	29.30	214	12.0	3.409?
126	- 18	0.952	0.77	28.25	200	10.0	2.884

### DISCUSSION.

The above experiments amply confirm the results of Mason regarding the differences which occur in the respiratory metabolism following administration of dihydroxyacetone and of glucose when the substances are given by the mouth. In fact, by taking air and blood samples at shorter intervals, the more rapid rise in the total metabolism, in the oxygen consumption and in the respiratory quotient is even more strikingly shown. Thus, within 15 minutes of the ingestion of 50 g. dihydroxyacetone by the normal individual, a rise of about 18 % in the total metabolism was observed, whilst after a similar amount of glucose a rise of only 3 % was obtained. Exp. 1 (a) is exceptional in that the respiratory quotient, high to begin with, rose to a maximum 15 minutes after the administration of glucose, but there was no corresponding rise in the oxygen consumption or in the metabolic rate. Overbreathing is the probable explanation of this result. In all other experiments, both in diabetics and normals, the R.Q. attained its maximum about 45 minutes after administration of glucose, as did also the total metabolism and the oxygen consumption. On the other hand, after dihydroxyacetone the various maxima were reached in 20 to 30 minutes. After this there was a rapid fall in all values. The experiments in which intravenous injection of this substance was employed show that more rapid absorption from the intestine is not sufficient to account for the observed results. Thus, after 20 g. glucose intravenously, the total

metabolism, oxygen consumption and respiratory quotient gradually rose, reaching their maximum in approximately half an hour. Simultaneously with the rise in the respiratory metabolism, the blood-sugar fell and had regained the normal level or fallen below it about the time that the metabolism was at its height. With dihydroxyacetone, on the other hand, the rise in the total metabolism, the oxygen consumption and the respiratory quotient was not only more marked than with glucose, but was immediate, attaining its maximum about the time that the injection was completed, after which there was a fall *pari passu* with the disappearance of dihydroxyacetone from the blood. The maximal increase in the total metabolism of normal individuals after glucose was about 16 %, while after dihydroxyacetone the metabolism rose to 57 % above the basal level. In the diabetic, the rise in the metabolism after glucose was much smaller than in the normal individual. Thus, in Exp. 6 (a) there was an increase of not more than 9 % in the total metabolism after 20 g. glucose intravenously, the respiratory quotient and oxygen consumption also showing only a slight rise. On the other hand, after injection of a similar amount of dihydroxyacetone, the resultant increase in metabolism was comparable to that met with in the normal individual—namely 57 %—the oxygen consumption and respiratory quotient showing a corresponding rise. Dihydroxyacetone, therefore, produced a much greater increase in the metabolism in the diabetic than did glucose in the normal (cf. Exps. 5 (a) and 6 (b)).

These facts would seem to indicate that dihydroxyacetone can either be directly and immediately oxidised or very rapidly changed into some substance—possibly an active form of dihydroxyacetone—which is oxidised with great ease. Glucose, on the other hand, would appear gradually to undergo some transformation prior to its oxidation. It is evidently this transformation which the diabetic has difficulty in effecting.

The above observations seem to be in accord with the hypothesis advanced in a previous paper [Kermack, Lambie and Slater, 1927]—namely, that the essential feature of insulin action is to facilitate the transformation of glucose into dihydroxyacetone, which is then directly oxidised.

Although dihydroxyacetone may be converted into glucose to a greater or lesser extent in the diabetic, especially after repeated small doses by the mouth, the sudden injection of a large quantity intravenously would cause a certain amount to be rapidly oxidised, thus raising the metabolism in the manner above described, before any considerable amount would have time to be converted into glucose.

This more rapid oxidation of dihydroxyacetone in the animal body is paralleled by its more ready oxidation *in vitro*, as compared with glucose. Thus Wind [1925] has found that in neutral phosphate solution it takes up atmospheric oxygen much more readily than laevulose, while glucose takes up hardly any oxygen at all. Experiments, which will be reported by one of us in detail in a future communication, have been performed which show that, in neutral phosphate solution at  $p_{\text{H}}$  7.4 and 37°, permanganate is much more

rapidly decolorised by dihydroxyacetone than by glucose and that dihydroxyacetone reduces methylene blue under anaerobic conditions whereas glucose does not. The latter observation is of interest in connection with the experiments of Ahlgren [1924], who found that the muscles of depancreatized animals, unlike those of normal animals, were unable to convert glucose into a hydrogen donor as tested by the methylene blue method, whereas addition of insulin restored the capacity to do so. Evidently some intermediary metabolite must have been formed from glucose under the influence of insulin which could act as a hydrogen donor. Dihydroxyacetone, which is a suggested intermediary, could at least fulfil this rôle.

It is now well established that increased metabolism of carbohydrates, whether brought about by ingestion of glucose or by injection of insulin, is accompanied by a fall in the inorganic phosphate of the blood, due in all probability to the formation of phosphoric acid esters of carbohydrate [Wigglesworth, Woodrow, Winter and Smith, 1923; Harrop and Benedict, 1924]. In view of this close connection between phosphate and carbohydrate metabolism together with the differences above described in the respiratory metabolism after administration of glucose and of dihydroxyacetone, there might naturally have been expected to be corresponding differences in the phosphate metabolism. Our protocols show that this is indeed the case. The curves of inorganic blood-phosphate follow closely those of the respiratory metabolism; the fall in inorganic blood-phosphate is more marked and more rapid after dihydroxyacetone than after glucose whether administered by the mouth or intravenously. Moreover, in the diabetic, where very little drop in the inorganic phosphate occurred after glucose, a marked fall followed the administration of dihydroxyacetone. It is difficult to reconcile these findings with those of Campbell, Fletcher, Hepburn and Markowitz [1926]. The fall in the inorganic blood-phosphate is not accounted for by increased excretion in the urine, since it was immediate after intravenous injection of dihydroxyacetone, while in some experiments little if any urine was eliminated during the whole period of the experiment. In other experiments the elimination of phosphate in the urine varied with the urine volume, rather than with the concentration of inorganic blood-phosphate.

These results would appear to indicate that administration of dihydroxyacetone causes the more rapid formation of a phosphoric acid ester than does the administration of glucose. The extremely rapid fall in inorganic phosphate following intravenous injection suggests that dihydroxyacetone may be directly esterified, forming a triosephosphate. Raymond [1925] and Kluyver and Struyk [1926] have proposed schemes in which triosephosphate figures as an intermediary in carbohydrate metabolism, without, however, bringing forward any experimental evidence in support of their views. Glucose, as suggested by Euler [1925] and Meyerhof [1926], is represented as being esterified to hexose-monophosphate, and the latter splits into one molecule of triose and one molecule of triosephosphate. Two molecules of triosephosphate then condense

to form one molecule of hexosediphosphate. This would seem to account more readily for the formation of hexosediphosphate than the explanation offered by Meyerhof, namely, that it is formed by the splitting of two molecules of hexosemonophosphate into hexosediphosphate (1 mol.) and lactic acid (2 mols.). It would be premature to speculate upon the nature or fate of the phosphoric esters actually involved in these processes, as this is a matter for further investigation, but the above experiments serve to draw attention to the possible importance of esters of dihydroxyacetone.

The experiments with laevulose show that this substance is utilised somewhat more easily than glucose, but not nearly as readily as dihydroxyacetone. Its effects upon metabolism appear to be intermediate between the two, but more closely approximating to those of glucose. In experiments upon the normal subject (including some not recorded in the above protocols) the R.Q. after oral administration of 50 g. dihydroxyacetone rose above unity, indicating that fat was being formed from carbohydrate. Respiratory quotients above unity were not obtained with either glucose or laevulose in the doses employed, namely, 50 g. Benedict and Carpenter [1918] have, however, shown that with 100 g. or after repeated administration of smaller quantities, respiratory quotients above unity may be obtained with laevulose but not with glucose. Dihydroxyacetone can therefore produce these high respiratory quotients with smaller doses than laevulose.

Only a small number of experiments upon the respiratory metabolism in diabetics following administration of laevulose have been recorded above, and these show that, in the doses employed, the effects were not very different from those obtained with glucose. It has, however, been demonstrated by numerous investigators [Minkowski, 1890; Lusk, 1915; Verzar, 1914; Tögel and co-workers, 1913; Johansson, Billström and Heijl, 1904] that with larger or repeated doses laevulose produces a much greater rise in the total metabolism and in the R.Q. than similar amounts of glucose. Joslin [1924] has even obtained respiratory quotients of unity or above in diabetics.

Laevulose, like dihydroxyacetone, is not only more easily oxidised in the animal body, but it is a better glycogen former than glucose. This is all the more remarkable in view of the fact that glycogen yields only glucose on hydrolysis. As far as can be ascertained there is no difference between the glycogen formed from glucose and that formed from laevulose [Cremer, 1902; Macleod, 1926]. Neither laevulose nor hexosediphosphate [Harden and Young, 1911; Young, 1911], which is probably a derivative of laevulose, can, therefore, presumably be the immediate precursors of glycogen. There would therefore seem to be some intermediary common to laevulose and to glucose from which glycogen is formed. Another fact pointing to the existence of a common intermediary is that in the diabetic laevulose is excreted for the most part as glucose [Minkowski, 1890]. It has been supposed that in the formation of glycogen from laevulose, the latter is converted into glucose through the common enol form of the hexoses. This would not, however, explain satisfactorily the greater



ease with which laevulose forms glycogen, nor why such a substance as dihydroxyacetone is a better glycogen former than glucose. The simplest hypothesis would seem to be that dihydroxyacetone is the intermediary common to laevulose and glucose and that glycogen is formed from it by condensation and polymerisation. In the diabetic, dihydroxyacetone would be converted into glucose and thus the increased elimination of the latter after administration of laevulose would be accounted for. It at first seems an objection to this assumption that laevulose, in spite of its being more readily oxidised in the animal body, even in diabetes, does not cause recovery from insulin hypoglycaemia as rapidly as does glucose [Noble and Macleod, 1923] or dihydroxyacetone. It is possible, however, that laevulose, being structurally more closely related to dihydroxyacetone than glucose, may be converted into dihydroxyacetone without the aid of insulin, and in this way its more ready utilisation by the diabetic, observed especially after large doses, would be accounted for. On the other hand, insulin may be supposed to be specifically adapted for the conversion of glucose (and mannose, whose structure closely resembles that of glucose) into dihydroxyacetone, a reaction occurring with difficulty, if at all, without it. In the presence of excess of insulin, however, as in animals suffering from experimental hypoglycaemia, the conversion of glucose into dihydroxyacetone might be even more rapid than the formation of the latter from laevulose. Hence, under these conditions, recovery would be more rapid with glucose than with fructose.

Finally, it may be noted that it is probable that dihydroxyacetone, like other ketoses, can exist in forms other than the stable variety [cf. Fischer and Taube, 1924] and we therefore cannot exclude the possibility that it is in fact a reactive form of the substance which is biologically important.

#### SUMMARY.

1. Dihydroxyacetone produces, both in diabetics and normals, a more rapid and more marked rise in the respiratory quotient, the oxygen consumption and the total metabolism than does glucose, whether administered intravenously or orally.

2. Dihydroxyacetone can be directly utilised by the diabetic. After intravenous injection of 20 g. over a period of 5 minutes, the immediate rise in the metabolism respiratory quotient and oxygen consumption is comparable to that obtaining in normal individuals.

3. The fall in the inorganic phosphate of the blood in both diabetics and normals is more rapid and more marked after dihydroxyacetone than after glucose, whether administered intravenously or orally. The curves of inorganic blood-phosphate follow closely those of the respiratory metabolism.

4. Laevulose produces effects intermediate between those of glucose and dihydroxyacetone, but more closely approximating to those of the former when small doses are employed.

5. Respiratory quotients above unity were obtained after oral administration of 50 g. dihydroxyacetone in normals, indicating the formation of fat from carbohydrate.

The above research was done under a grant to one of us (C. G. L.) from the Medical Research Council. Part of the expenses were defrayed by a grant from the Moray Fund.

## REFERENCES.

- Ahlgren (1924). *Klin. Woch.* **3**, 1158; *Chem. Zentr.* **2**, 1007.  
Benedict and Carpenter (1918). *Carnegie Inst. Rep.* **261**, 243, 253.  
Briggs (1922). *J. Biol. Chem.* **53**, 13.  
Campbell, Fletcher, Hepburn and Markowitz (1926). *J. Biol. Chem.* **67**, 57.  
Cremer (1902). *Ergebn. Physiol.* **1**, 803.  
Euler (1925). *Chemie der Enzyme*.  
Fischer and Taube (1924). *Ber. deutsch. chem. Ges.* **57**, 1502.  
Harden and Young (1911). *Biochem. Z.* **32**, 173.  
Harrop and Benedict (1924). *J. Biol. Chem.* **59**, 683.  
Isaac and Adler (1924). *Klin. Woch.* **3**, 1208.  
Johansson, Billstrom and Heipl (1904). *Skand. Arch. Physiol.* **16**, 263.  
Joslin (1924). *Treatment of Diabetes*, p. 291.  
Kermack, Lambie and Slater (1926). *Biochem. J.* **20**, 486.  
--- (1927). *Biochem. J.* **21**, 40.  
Kluyver and Struyk (1926). *Naturwiss.* **14**, 882.  
Lusk (1915). *J. Biol. Chem.* **20**, 555.  
Macleod (1926). *Insulin and Carbohydrate Metabolism*, p. 143.  
Mason (1926, 1). *Canad. Med. Assoc. J.* **16**, 367.  
— (1926, 2). *J. Clin. Invest.* **2**, 521, 533.  
Meyerhof (1926). *Naturwiss.* **10**, 196; **14**, 1176; **32**, 756.  
Minkowski (1890). *Arch. exp. Path. Pharm.* **26**, 371.  
Noble and Macleod (1923). *Amer. J. Physiol.* **64**, 547.  
Rabinowitch (1925). *J. Biol. Chem.* **65**, 55.  
Raymond (1925). *Proc. Nat. Acad. Sci.* **11**, 622.  
Togel, Brezina and Durig (1913). *Biochem. Z.* **50**, 319.  
Verzar (1914). *Biochem. Z.* **66**, 75.  
Wigglesworth, Woodrow, Winter and Smith (1923). *J. Physiol.* **57**, 417.  
Wind (1925). *Biochem. Z.* **159**, 58.  
Young (1911). *Biochem. Z.* **32**, 178.

# LXXIX. THE PURIFICATION AND PROPERTIES OF INSULIN.

By FRANK DICKENS, EDWARD CHARLES DODDS,  
WILFRID LAWSON AND NOËL FRANCIS MACLAGAN.

*From the Biochemical Department, the Middlesex Hospital, London.*

*(Received April 27th, 1927.)*

It is a matter of common experience that, whilst the purification of crude insulin to a degree of activity represented by a unit of approximately 0.1 mg. presents few difficulties, attempts to exceed this to any great extent usually result in a considerable loss. The methods employed for this purpose may be grouped into two main classes: (a) precipitation at the isoelectric point, and (b) the use of protein precipitants.

With the former method it is usual to find that after repeated precipitation the activity is not further increased, moreover, considerable losses accompany each precipitation. This fact is frequently referred to by various workers, *e.g.* Shonle and Waldo [1924], and although in some cases material has been prepared which greatly exceeds the degree of activity represented by the unit mentioned above, such methods of purification are not ideal for the preparation of insulin in quantities sufficient either for clinical use or for experimental purposes, owing to the small fraction of the original number of units which is recovered in the final product after several precipitations.

The use of the second group of methods, whilst it does not generally involve the loss of active material which accompanies isoelectric precipitation, results in little purification beyond the limits referred to above. Moreover, it is frequently impossible to purify by this means certain specimens of insulin, notably those prepared from pancreas in which, owing to insufficient freezing, tryptic action has occurred, so as to obtain material the unit of which is below 1 mg. or even more. The following investigations were undertaken with a view to determining the reasons underlying the failure by these methods.

In order to characterise the group of protein fission products to which insulin belongs it was thought that the application of the recently described method of Wasteneys and Borsook [1924] would prove to be of value. These workers showed that if trichloroacetic acid were added to a solution containing a mixture of proteins and their disintegration products in sufficient quantity to make up the concentration in the solution to 2 %, then proteins and meta-proteins alone were precipitated. On applying this method to insulin solutions, it was found that the whole of the potency was precipitated under these con-

ditions. The supernatant fluid from this precipitation was always found to give a further precipitate on the addition of picric acid, even when insulin of considerable purity was used. In order to test the effect of this 2 % trichloroacetic acid precipitation quantitatively it was necessary to devise some method whereby the insulin could be recovered from the trichloroacetic acid precipitate, and it was found that if this were dissolved in dilute acid, the activity was completely precipitated by the addition of sodium chloride. This precipitate was free from trichloroacetic acid, and could easily be converted into the hydrochloride.

In this way, a quantitative recovery of the activity results, together with considerable purification. If a specimen of insulin which has been through this process is subjected to treatment with 2 % trichloroacetic acid again, it is found that the whole is precipitated and that the supernatant fluid yields at most a trace of picrate, and that therefore nothing is to be gained by repeating this treatment. This experiment would show, according to Wasteneys and Borsook, that the active fraction of insulin must be associated with the groups of protein or metaprotein, but the application of these views to the question of the constitution of insulin is discussed later. It is clear, however, that the greater part of the material precipitated by 2 % trichloroacetic acid must consist of proteins or metaproteins, and this suggested that physico-chemical means might be applied to the problem of further purification.

Hardy [1905] showed that at or near their isoelectric point globulins can be dissolved by neutral salt solutions. More recently Cohn [1924] has shown that the degree of such solubility is a characteristic property of each individual protein, and it was thought that the application of this principle to insulin might yield valuable results. Accordingly an isoelectric precipitate of insulin was washed with solutions of potassium chloride, both the material dissolved and the insoluble residue being converted to hydrochloride. Tests upon rabbits demonstrated that the active fraction is relatively insoluble in dilute salt solution, whereas some of the inactive portion dissolves more readily; thus purification has been effected.

Hardy also showed that bivalent and tervalent anions are capable of holding in solution more globulin than are univalent anions. After some preliminary trials it was decided to investigate the action of oxalates and citrates upon insulin, but it was clear that an experiment similar to the one quoted above with sodium chloride could not be performed, because these salts are in themselves weak buffers, the solutions having a  $p_H$  of 7 or over, and if the isoelectric precipitate of insulin is washed with a solution of potassium oxalate, then the precipitate dissolves, owing to the fact that the  $p_H$  rises above that of the isoelectric point.

The effect of adding a  $N/8$  solution of potassium oxalate to a solution of insulin hydrochloride (which is strongly acid) was then observed. On adding such a solution drop by drop it was found that a precipitate began to appear when the  $p_H$  had reached about 3.7, and reached a maximum at about  $p_H$  4,

and then the solution began to clear as the  $p_H$  rose. The formation of a maximum precipitate between  $p_H$  3.7 and 4.2, instead of at the normal isoelectric point ( $p_H$  4.5-5.0 with this particular material), appeared to be capable of explanation by the work of Michaelis and Szent-Györgyi [1920], who showed that the isoelectric point of certain proteins can be definitely lowered by salts. The experiments described confirm this, in the case of insulin, and the lowering of the isoelectric point is shown to depend upon the concentration of oxalate present. Great purification was obtained in this manner with little or no loss of activity.

In the light of the foregoing experiments it appears that the following explanation may be advanced to account for this phenomenon. The addition of a weak buffer of potassium oxalate slowly raises the  $p_H$  of the solution and at the same time lowers the isoelectric point of the insulin until a precipitate is produced. The potassium oxalate holds in solution inactive fractions which will be precipitated during the course of an ordinary isoelectric precipitation.

This type of method has certain objections both from a practical and a theoretical aspect. In the first place, it is difficult to determine the end-point of the titration except by centrifugation after each small addition of oxalate, and, secondly, since the amount of inactive material held in solution must vary with the quantity of oxalate present, it is often impossible to obtain a sufficiently high concentration of oxalate without raising the  $p_H$  to a level above the isoelectric point, thus dissolving some of the active portion. A method was therefore devised in which the insulin solution could be added to a buffer solution of suitable  $p_H$  value which contained sufficient oxalate to hold the relatively greater quantities of inactive material in solution. It was found that to produce a  $p_H$  of 4, the point at which maximum precipitation occurs in the above experiment, 100 cc. of potassium oxalate solution must be added to 20 cc. of oxalic acid solution. The  $p_H$  of the solution of insulin hydrochloride was adjusted to 4, and it was then run into two volumes of the above buffer solution with vigorous stirring. A precipitate was produced which was found to contain all the original potency in a much higher concentration, showing that a large amount of inactive material had been removed.

By combining the trichloroacetic acid precipitation, the acid and salt precipitation and the oxalate precipitation, a very efficient purification method was obtained. Similar results are obtained when sodium citrate is used in place of potassium oxalate. The details of these investigations are given in the experimental section.

In the above examples we have employed solutions of approximately  $p_H$  4, since, for the particular specimens of insulin used (isoelectric point at or about  $p_H$  5), this represents the optimum condition. We have observed, however, that many specimens of insulin hydrochloride possess an isoelectric point much higher than that recorded hitherto. This applies more especially to some specimens of material prepared by the acetone-picric acid process, the isoelectric point of which is frequently at  $p_H$  5.9, or even higher. If such solutions

be brought to  $p_H$  5.7 there is often no sign of precipitation. In this case, the oxalate mixture at  $p_H$  4 is clearly too acidic and only a small fraction of the activity is precipitated on mixing the solutions at this hydrogen ion concentration. By first performing an isoelectric precipitation on a portion of the solution and determining the hydrogen ion concentration at maximum precipitation this difficulty can be avoided. In such cases the reactions of the oxalate solution and the insulin solution should be brought to a higher  $p_H$  value (approximately 5.0) before mixing, or the original method of running the solution of potassium oxalate into the solution of insulin hydrochloride to maximum precipitation may be employed.

#### EXPERIMENTAL.

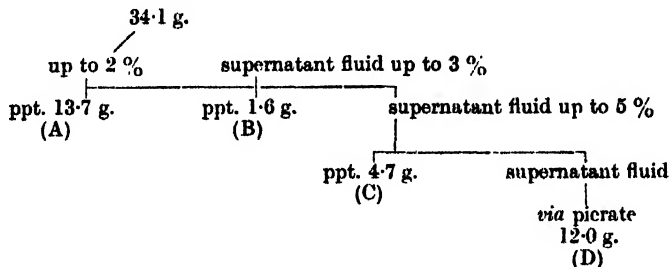
##### *The precipitation of insulin by trichloroacetic acid.*

(a) *Effect of concentration.* Crude insulin hydrochloride (19.7 g.) was dissolved in water to give a 1.5 % solution. A 10 % solution of freshly distilled trichloroacetic acid was added drop by drop with constant stirring until the concentration of the acid was 1 %. There was no precipitation. A further measured quantity of trichloroacetic acid was added to bring the concentration up to 1.5 %. The precipitate which was obtained was allowed to stand for 2 hours and was then centrifuged as completely as possible. Although this operation was performed at a high speed, the supernatant fluid was still faintly turbid. It was decanted from the precipitate and trichloroacetic acid was added to it to bring the concentration up to 2 %. A further precipitation resulted, and after centrifugation the supernatant fluid was perfectly clear. The precipitates were dissolved in  $N/10$  hydrochloric acid and, on the addition of half the volume of saturated sodium chloride solution to each, a precipitate formed. This was collected in the centrifuge, dissolved in water, and the material was obtained from the solution in the usual way by precipitation as picrate and conversion to the hydrochloride [Dudley and Starling, 1924]. The two fractions thus obtained weighed 1.86 and 2.84 g., and standardisation showed that they contained 1230 and 2840 units respectively. The initial material had 4500 units. This experiment shows that by means of the precipitation 15.0 g. of almost completely inactive material had been removed. It is also clear that, whereas a 1.5 % concentration of trichloroacetic acid is insufficient for complete precipitation of the activity, 2 % trichloroacetic acid causes almost the whole of the activity (90 % in this example) to be precipitated. The addition of further quantities of 10 % trichloroacetic acid brought about the precipitation of inactive material.

In the above example very crude insulin hydrochloride was used. Similar experiments with highly purified material have also shown that the activity is completely precipitated by 2 % trichloroacetic acid.

(b) *Fractional precipitation with trichloroacetic acid.* An experiment performed in a manner similar to that described above was carried out, using

concentrations of trichloroacetic acid of 2, 3 and 5 %. The precipitates in each case were converted to hydrochloride. The results are shown in the following table. The initial weight of material (34.1 g.) contained 34,000 units. Precipitate A below, the unit of which proved to be 0.44 mg., contained 31,000 units.



Of these fractions A is the only one which behaves as a typical protein or metaprotein, *e.g.* it is precipitated at the isoelectric point, completely precipitated by half saturation with ammonium sulphate and by copper sulphate. B gives only a very faint turbidity at the isoelectric point, whilst solutions of C and D remain perfectly clear, and all three yield a further precipitate on full saturation with ammonium sulphate and are not precipitated by copper sulphate. The precipitates of B, C and D, obtained on adding potassium ferrocyanide and acetic acid, clear on warming, indicating the presence of primary proteose in these fractions.

The distribution of amino- to total nitrogen in these fractions is shown in Table I. It will be seen that there is a gradual rise in the ratio of amino- to total nitrogen with each fraction.

Table I.

Trichloroacetic acid	Amino-N %	Total N %	Amino-N Total N %
(A) Up to 2 %	0.82	14.06	5.8
(B) 2 to 3 %	0.94	14.65	6.4
(C) 3 to 5 %	0.98	14.80	6.6
(D) Not precipitated by 5 % trichloroacetic acid	1.41	15.00	9.4

*Solubility of the isoelectric precipitate in salt solutions.*

(1) *Potassium chloride.* Qualitative experiments having shown that the inactive fractions, separated from insulin during the course of purification by various means, tend to dissolve much more readily when potassium and sodium chloride solutions are added to their isoelectric precipitates than does the active portion, the following roughly quantitative experiment was performed in order to estimate the partition of active and inactive material in solution and precipitate. Crude insulin hydrochloride (110 mg.) was isoelectrically precipitated by the addition of *N*/100 potassium hydroxide solution (6.5 cc.); the precipitate was collected in the centrifuge, the supernatant fluid

discarded, and the precipitate well stirred with  $N/2$  potassium chloride solution. A portion dissolved and was recovered in the usual way from the supernatant fluid obtained after centrifugation. The precipitate was also converted into the picrate and hydrochloride. The undissolved portion weighed 16 mg. and contained 100 units. The solution yielded 21 mg. hydrochloride containing 30 units. The solubility of the isoelectric precipitate in water alone was shown to be negligible under the same conditions by a control experiment.

(2) *Potassium oxalate.* To a solution of insulin hydrochloride (4.00 g. = 34,000 units) in water (50 cc.) a  $N/2$  solution of potassium oxalate was run in with stirring. The addition of 10.0 cc. produced the first permanent turbidity. The initial reaction of the solution of hydrochloride, as determined by the hydrogen electrode, was  $p_H$  2.35, and after addition of 24.8 cc. of oxalate this had risen to  $p_H$  3.46. Precipitation was now complete, and precipitate and supernatant fluid were separately converted to picrate and then to hydrochloride. The precipitate after washing and drying weighed 1.14 g. and contained 28,400 units. In this experiment considerable activity was contained also in the supernatant fluid which yielded 1.79 g. hydrochloride containing 3670 units. (In this case the portions insoluble in acid alcohol, during the preparation of the hydrochloride from the picrate, were rejected.)

This experiment shows that oxalate may be used in the same way as potassium chloride for dissolving a considerable portion of material of low activity. Repetition of the process gave a final product which could not be further precipitated by this means without considerable loss. In these later experiments better results were obtained by using more dilute solutions of hydrochloride and of oxalate. Finally, for reasons to be discussed later,  $N/8$  potassium oxalate was added to a 1.5 % solution of the hydrochloride. In this way, after four more precipitations, 0.483 g. of hydrochloride was obtained, the unit of which, obtained according to the method laid down in the League of Nations Report [1926], proved to be 0.016 mg., a total recovery of 30,000 units after five precipitations in all. A loss of 10 % on each stage would thus have resulted in a final yield of only 60 %, whereas actually the recovery was almost quantitative. Isoelectric precipitation, carried out by adding  $N/10$  sodium hydroxide to the hydrochloride in the usual way, resulted in a loss of approximately 25 % of the activity in each precipitate.

The above example shows that when used for purifying insulin which is already of the order of 0.1 mg. per unit this type of process gives satisfactory purification and yields. Even for this purpose, however, it is troublesome to determine the end-point when complete precipitation is reached, this necessitating repeated centrifugation and addition of further small quantities of oxalate. Moreover, when cruder specimens of insulin are used losses occur. In order to obtain precipitation under uniform conditions the solution was first adjusted by the addition of dilute alkali to  $p_H$  4 ( $\pm$  0.05), experiments having shown that this is an average figure with this material for precipitation in the presence of oxalate. In the following experiment this solution contained 1.0 g.



of crude insulin (= 1250 units) in 25 cc. It was run into 50 cc. of a solution of potassium oxalate and oxalic acid containing five volumes of  $N/8$  oxalate to one volume of  $N/8$  oxalic acid. The  $p_H$  of this solution was 4.05. Although the hydrogen ion concentration remained constant, on running in the insulin solution a precipitate at once began to form. This was collected and converted to hydrochloride, when it weighed 0.264 g. and contained 1200 units. The supernatant fluid, converted to hydrochloride through the picrate, weighed 0.622 g., and the unit was considerably above 2 mg.

(3) *The concentration of oxalate.* These results now made it possible to investigate the optimum concentration of oxalate which should be used, the hydrogen ion concentration remaining constant at  $p_H$  4, in order that a maximum quantity of inactive material might be held in solution, whilst the activity should be found in the precipitate. For this purpose, oxalic acid-potassium oxalate solutions were prepared such that all were at  $p_H$  4 ( $\pm 0.05$ ), the concentration of oxalate ions being respectively  $N/4$ ,  $N/6$ ,  $N/8$  and  $N/10$ . A solution of crude insulin (3.75 g. = 4300 units) in 250 cc. water was adjusted to  $p_H$  4.02. Equal portions of 60 cc. each were then run into 120 cc. of the above oxalate solutions. After standing for 2 hours, the mixtures were centrifuged, and the precipitates converted into hydrochloride. The results are shown in the following table:

Final dilution of oxalate	$p_H$ after addition of insulin	Wt. of hydrochloride g.	Unit in mg.	Total units recovered %
$N/6$	3.99	0.174	0.17	100
$N/9$	4.00	0.148	0.14	100
$N/12$	3.99	0.113	0.11	100
$N/15$	3.98	0.062	0.15	40

From this it is clear that between  $N/12$  and  $N/15$  there is an abrupt transition, and that at the lower concentration more than half of the activity remains unprecipitated. The use of higher final concentrations than  $N/12$  is to be avoided, however, since a greater proportion of inactive substance accompanies the precipitate, whilst by the use of a concentration of  $N/12$  all the activity is carried down with a minimum of other material. The necessity of allowing the mixture to stand for 2 hours in order to ensure complete precipitation should be emphasised.

The effect of concentration of oxalate upon the hydrogen ion concentration at which precipitation of insulin from solution occurred was investigated. The specimen of insulin used had a normal isoelectric point of  $p_H$  5.1.

A solution of insulin hydrochloride (1.00 g.) in water (40 cc.) was treated with  $N/10$  sodium hydroxide solution until maximum precipitation occurred (10.2 cc.). The suspension of isoelectric insulin was well shaken and divided among a series of tubes in equal portions. To each of these was added an equal volume of a solution of oxalic acid of double the strength of that finally required. The precipitates dissolved immediately and potassium oxalate of the required normality was run into the clear solutions until precipitation occurred. The

hydrogen ion concentrations were then determined, and the results are shown in Fig. 1.

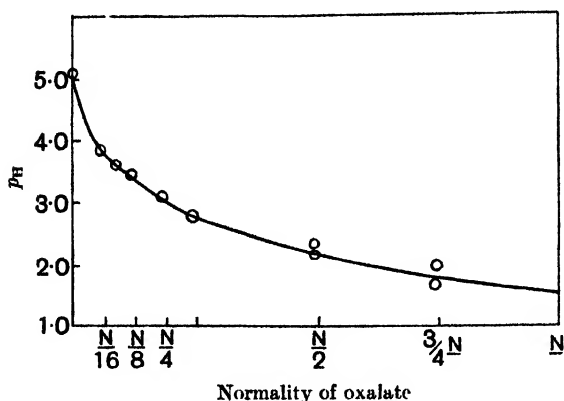


Fig. 1.

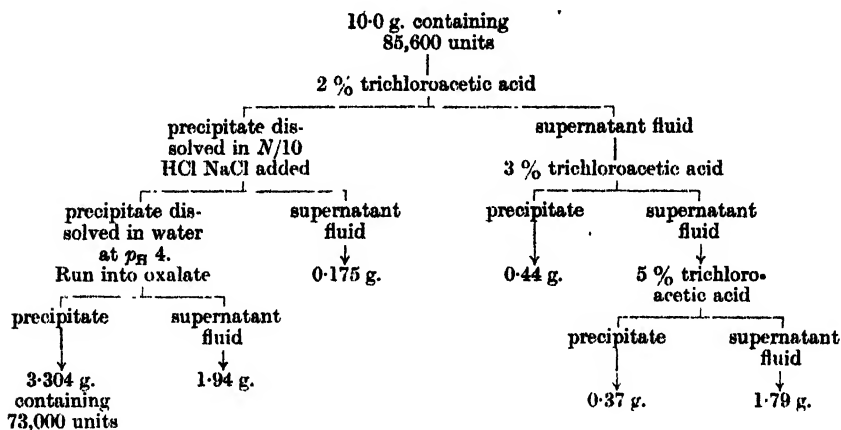
*Complete purification process.*

The three operations already described were now combined. The following is a typical example. 10.0 g. of insulin containing 85,600 units were dissolved in water and the solution centrifuged to remove a small quantity of insoluble material. The clear solution was diluted to 667 cc. and to this was added with stirring a 10 % solution of trichloroacetic acid (167 cc.). The precipitate settled fairly rapidly, leaving the supernatant fluid only faintly opalescent. After 2 hours the main portion of this was removed by means of a syphon, and the precipitate was collected in the centrifuge. The supernatant fluid gave further precipitates on bringing the concentration of trichloroacetic acid successively up to 3 % and 5 %. These precipitates were collected separately, and these and also the material remaining unprecipitated by 5 % trichloroacetic acid were converted to hydrochloride through the picrate.

The precipitate obtained by 2 % trichloroacetic acid was not converted directly to hydrochloride, but, while still moist, it was dissolved in  $N/10$  hydrochloric acid (260 cc.), and to this 130 cc. of saturated sodium chloride solution was run in with good stirring. The precipitate was centrifuged, and the supernatant fluid was neutralised, and, after dilution to avoid precipitation of sodium picrate, the picrate was precipitated. This was converted to hydrochloride also. Meanwhile the salt precipitate was dissolved in water (200 cc.), its reaction was adjusted to  $p_H$  4.0, and the solution was run with good stirring into 400 cc. of the  $N/8$  oxalate mixture ( $p_H$  4.05). The precipitate which formed was collected in the centrifuge after 2 hours, the precipitate dissolved in the minimum quantity of dilute hydrochloric acid, the solution diluted to about 200 cc., and, after neutralisation of the excess acid, half its volume of saturated picric acid solution was added. This picrate was well washed in the centrifuge with dilute picric acid solution, and was then converted into the hydrochloride.

Similarly, the material in the supernatant oxalate solution was also converted to picrate and hydrochloride. The weights of hydrochloride obtained at each stage are given in Table II.

Table II.



It sometimes happens that, owing to much salt remaining adherent to the precipitate, on adjusting the hydrogen ion concentration to  $p_H$  4 a precipitate occurs. This may contain a considerable proportion of the activity. It is collected in the centrifuge and redissolved in dilute acid, when the solution may usually be adjusted to  $p_H$  4 without any precipitation occurring. Both the solutions are then run into the oxalate solution separately in the usual way, and the final product is obtained by combining the two precipitates. If the purification by means of oxalate buffer be repeated there will be no precipitate at  $p_H$  4 on adjusting the reaction of the solution prior to oxalate precipitation.

Table III shows the results of a series of experiments where the full procedure was followed.

Table III.

Initial weight g.	Initial unit mg.	Total initial units	No. of oxalate precipita- tions	Weight recovered g.	Final unit mg.	Total units recovered	Purifica- tion ratio
130	6.0	21,600	1	9.0	0.5	18,000	12
136	1.05	129,500	1	15.0	0.125	120,000	8.4
30.5	1.0	30,500	2	2.70	0.09	30,000	11.1
10.0	0.116	85,600	1	3.30	0.045	73,000	2.3
			2	2.53	0.035	72,400	3.3
			5	1.82	0.030	60,600	3.9

*Use of sodium citrate.* To a solution of insulin hydrochloride (1.0 g. = 1000 units) in 67 cc. water, *N*/10 solution of sodium citrate was added drop by drop. The initial  $p_H$  values of the hydrochloride and citrate solutions were 2.4 and 7.3 respectively. After 16 cc. had been added a permanent turbidity appeared, the reaction being  $p_H$  4.5. Complete precipitation was obtained after adding 28 cc. of citrate, when the hydrogen ion concentration had fallen to  $p_H$  5.05.

The precipitate was collected in the centrifuge, and both the precipitate and the material contained in the supernatant fluid were converted into hydrochloride in the usual way. The precipitate yielded 0.254 g. of substance containing 960 units. From the supernatant fluid the weight obtained was 0.52 g., the unit of which was approximately 8 mg. From this experiment it is clear that citrate acts similarly to oxalate in holding in solution a large proportion of inactive material.

*Qualitative tests.*

The following tests were performed on specimens of insulin the rabbit units of which lay between 0.016 and 0.03 mg. Solutions containing 2 mg. or more per cc. were used. The Pauli reaction was strongly positive with all the specimens tested, whether in the usual way or after destruction of the tyrosine by nitration by Brunswick's method [cf. Shonle and Waldo, 1924]. The biuret, Millon and xanthoproteic reactions were also always positive. The reaction for tryptophan and the Molisch reaction were negative. If carefully neutralised before performing the test, all specimens gave a positive ninhydrin reaction. Jaffé's reaction with alkaline picric acid was positive, which may indicate, according to Abderhalden, the presence of diketo-piperazine rings. Although these experiments were performed quantitatively whenever possible, in no case was the intensity of the reaction proportional to the activity of the preparations used.

*Heat coagulation.* A solution of purified insulin hydrochloride in 1.5 % aqueous solution is not coagulated if the temperature is slowly raised to 100°. When a suspension of the isoelectric precipitate is heated in the same way it coagulates, the activity being contained almost completely in the precipitate. The coagulated precipitate so obtained may be dissolved in dilute acid, showing that this is not an irreversible coagulation. The precipitate obtained on adding sodium chloride solution to a solution of insulin hydrochloride, when treated similarly, is irreversibly coagulated.

*Dialysis.* A solution of insulin hydrochloride (0.500 g. containing 2000 units) previously purified by precipitation with 2 % trichloroacetic acid, as described above, was dialysed in a collodion thimble against *N*/10 hydrochloric acid for 18 hours. At the end of this time the level of liquid inside the thimble was higher than that outside, and the outer liquid had taken on a yellowish tinge. Both the inside and outside fluids were neutralised, and the dissolved material was precipitated as picrate. It was found that 0.147 g., almost a third of the total weight of substance, had passed through the membrane, and when tested upon rabbits this was found to contain approximately 15 % of the total initial activity. A similar result is reported by Shonle and Waldo [1924]. The dialysate is completely precipitated by 2 % trichloroacetic acid and there was only a slight difference in the ratio of amino- to total nitrogen, determined first on the original material and secondly on the dialysate, as shown in Table IV. No difference was observed in the unit of the dialysate and dialysed solution, when material which had been previously precipitated

Table IV. *Dialysis.*

	Weight in g.	Amino-N %	Total N %	$\frac{\text{Amino-N}}{\text{Total N}}$ %
Inside	0.259	0.845	14.4	5.85
Outside	0.147	0.98	14.2	6.9
Original material	0.500	0.817	14.1	5.8

with 2 % trichloroacetic acid was employed. After prolonged dialysis (5 days) the whole of the material was found to pass through a similar membrane, the outer liquid being replaced by fresh distilled water at 24-hourly intervals.

None of the material passed through a parchment membrane (Schleicher and Schüll) when treated in an exactly similar way.

*Precipitation by tungstic acid.* A solution of insulin hydrochloride was precipitated by tungstic acid, as in the Folin method of blood analysis, the whole of the activity being carried down.

*Effect of heat on the trichloroacetic acid precipitate.* It was observed that on warming a suspension in 2 % trichloroacetic acid of the trichloroacetic acid precipitate, which had previously been washed in the centrifuge with 2 % trichloroacetic acid, the suspension cleared slightly. The solution was warmed to 60° and was filtered through analytical filter paper in a jacketed funnel maintained at 60°. The clear filtrate, on cooling, became turbid. This was centrifuged, and the precipitate was converted to hydrochloride in the usual way. This precipitate contained 6 % of the weight of the original specimen and approximately 6 % of the initial activity. Experiments on this point are being continued. Similar results were obtained on heating a suspension of the precipitate, caused by the addition of sulphosalicylic acid to insulin solutions.

An attempt was made to prepare from our highly purified specimens insulin in a crystalline state, following the method of Abel [1926], but without success.

#### *Quantitative determinations.*

*Inorganic constituents.* By the process described it is possible to obtain material which leaves a very slight ash on incineration. 4.663 mg. (unit = 0.03 mg.) yielded 0.004 mg. ash.

The *sulphur* content of insulin preparations of varying degrees of activity was determined by Carius' method. The results are shown in Table V.

Table V.

Unit (mg.)	1.5	1.5	1.0	0.3	0.25	0.2	0.15	0.06	0.03
Sulphur %	1.7	2.0	1.9	1.6	1.5	1.9	2.0	2.0	1.55

The sulphur content shows but slight variation even in specimens of insulin differing in activity in the ratio of 50 to 1.

*Nitrogen.* The amino-nitrogen and total nitrogen content of insulin hydrochloride has been determined in a number of cases. The values obtained vary only slightly for specimens of very different degrees of purity, the units of specimens investigated lying between 0.15 mg. and 0.016 mg. The total nitrogen

varied from 13.7 to 15.0 %, the ratio of amino-nitrogen to total nitrogen from 5.33 to 6.9 %. All these preparations had previously been precipitated by 2 % trichloroacetic acid. These results are similar to those obtained by Scott [1925]. We have also analysed by van Slyke's method a specimen of insulin the unit of which was 0.03 mg. We have added for comparison the figures obtained by us on a specimen of very crude insulin hydrochloride containing 1.5 mg. of solids per unit. The results are given in the following table:

Unit	1.5 mg.	0.03 mg.
Ammonia-N	9.43	9.59
Melanin-N	1.29	0.52
Cystine-N	6.06	5.93
Arginine-N	15.28	9.37
Histidine-N	2.93	4.29
Lysine-N	5.01	0.21
Amino-N (non-basic)	52.64	67.61
Non-amino-N (non-basic)	10.48	5.14
Total N (sum)	103.1	102.6

Apart from a marked difference in the cystine nitrogen and a reduction in the lysine content in our purified material, there is no very striking difference between the figures obtained by Scott and by us, although the material analysed by us was approximately four times purer than his. These results show the danger of speculating on the "purity" of insulin preparations on account of similarity between such analyses; see, for example, the conclusions of Somogyi, Doisy and Shaffer [1924].

#### SUMMARY.

1. Insulin is completely precipitated with the protein and metaprotein class, following the analytical method of Wasteneys and Borsook.
2. On this basis a method of purification has been developed, depending on the action of salt solutions at the isoelectric point, which can be used to produce insulin of a very low physiological unit (about 0.02 mg.).
3. The results of various qualitative tests on the purified product are described.
4. Analytical results on specimens of insulin of varying degrees of purity are given.
5. The bearing of these observations on the probable nature and constitution of insulin is discussed.

#### REFERENCES.

- Abel (1926). *Proc. Nat. Acad. Sci.* **12**, 132.  
 Cohn (1924). *J. Biol. Chem.* **59**; *Proc. Amer. Soc. Biol. Chem.* iv.  
 Dudley and Starling (1924). *Biochem. J.* **18**, 147.  
 Hardy (1905). *J. Physiol.* **33**, 251.  
 League of Nations Report (1926). The Biological Standardisation of Insulin (Geneva).  
 Michaelis and Szent-Györgyi (1920). *Biochem. Z.* **103**, 178.  
 Scott (1925). *J. Biol. Chem.* **65**, 601.  
 Shonle and Waldo (1924). *J. Biol. Chem.* **58**, 732.  
 Somogyi, Doisy and Shaffer (1924). *J. Biol. Chem.* **60**, 31.  
 Wasteneys and Borsook (1924). *J. Biol. Chem.* **62**, 1.

# LXXX. A NOTE ON THE WEIGHT OF THE RAT DURING GESTATION.

By GLADYS ANNIE HARTWELL.

*From the Physiological Laboratory, Household and Social Science Department,  
King's College for Women, Kensington, London, W. 8.*

*(Received April 28th, 1927.)*

MANY observers have demonstrated the now well-established fact that a nursing mother can sacrifice her own tissues for the benefit of her suckling young, and it has been shown that a rat can lose as much as one-third of her weight during the lactation period [Hartwell, 1922]. The loss in weight of the mother is correlated with a poorness in *quality* of her diet, and a problem which at once presents itself is: does a similar sacrifice of the maternal organism occur during gestation?

The earlier research work on pregnancy was mainly along metabolic lines and was based on an analysis of excreta, the N balance being the primary consideration, while the weight of the animal was rarely recorded. A summary of this earlier work is given by Marshall [1922] who points out that the majority of the experiments were made before the vitamins were known and therefore are of little value now. According to the same writer, Cramer finds that rats fed on a diet of bread, rice and maize maintained their weight at the end of pregnancy, but when the diet was supplemented by yeast preparations, the mother always showed a considerable gain in weight during this period.

Feldman [1920], summing up the chief points of Bar's researches, regards pregnancy as a "state of symbiosis" and considers that there should be no loss to the maternal organism, provided the diet is satisfactory, but if it is not, then the results are disastrous, both for the mother and her foetus. In some earlier experiments [Hartwell, 1924, 2] when the rats were receiving a notably poor diet, it was found that there was little or no loss in the mother's weight at the end of gestation, so it was thought advisable to work out any available data in this respect.

## EXPERIMENTAL.

### (a) *Methods.*

The rats from which the data given in this paper have been collected are piebald animals bred in the laboratory. In most of the experiments 6 ♀ and 6 ♂ were used and were kept in a cage together; the method of weighing, feeding, etc., has been fully described in previous papers [Hartwell, 1926 and earlier]. The number of young in each litter was reduced to six, and the doe was returned to the bucks at the end of the suckling period (21 days); she

was not allowed a "rest" as given by other workers [Smith and Chick, 1926]. According to Donaldson [1924] and Greenman and Duhring [1923] the gestation period is 22 days, but 23 days was taken in these experiments, because it has been found that litters are never born before 23 days after mating. The difference in weight at the beginning and end of gestation given in the table was obtained as follows: the mother rat was weighed the day after the litter was born and the beginning of pregnancy was taken as approximately 23 days before the birth of the young.

### (b) Diets.

The proportions used in the different foods were:

- I a. 30 g. white bread, 3 g. butter, 0.7 g. salt mixture, water [Hartwell, 1924, 1 and 2].
- I b. 30 g. brown bread, 3 g. butter, 0.7 g. salt mixture, water [Hartwell, 1924, 2].
- II. 30 g. white bread, 12 g. food casein, 3 g. butter, 0.7 g. salt mixture, water [Hartwell, 1924, 3].
- III a. 30 g. white bread, 3 g. food casein, 3 g. butter, 0.7 g. salt mixture, water [Hartwell, 1924, 3].
- III b. 30 g. white bread, 3 g. ash-free casein, 3 g. butter, 0.7 g. salt mixture, water.
- IV. 30 g. white bread, 3 g. gluten, 3 g. butter, 0.7 g. salt mixture, water [Hartwell, 1924, 3].
- V. 100 g. oatmeal, 2.88 g. salt mixture, 14 g. butter, 500 cc. water [Hartwell, 1926].
- VI. 100 g. boiled potato, 5 g. butter, 1 g. salt mixture, 25 cc. water [Hartwell, 1927].
- VII. 15 g. white bread, 4.3 g. dried milk, 36 cc. water [Hartwell, 1925, 2].
- VIII. 15 g. white bread, 36 cc. cow's milk [Hartwell, 1925, 2].
- IX. 15 g. white bread, 36 cc. diluted evaporated milk [Hartwell, 1925, 2].
- X a. Synthetic diet [Hartwell, 1925, 1].
- X b. The same as X a, but containing more marmite.
- XI. Controls fed on kitchen scraps supplemented by bread and milk.

### RESULTS AND COMMENTS.

A striking feature is the almost constant gain in weight of the mother during gestation. The actual differences in weight are summed up in the following table.

Diet	Number of rats	Number of litters born	Weight of does at beginning and end of gestation			Average		Gain		Loss	
			Number of times weight gained	Number of times no loss or gain	Number of times weight lost	gain g.	loss g.	max. g.	min. g.	max. g.	min. g.
I a (Exp. 1)	3	9	9	0	0	18	0	37	9	0	0
(Exp. 2)	6	11	11	0	0	20	0	35	5	0	0
(Exp. 3)	6	12	12	0	0	23	0	37	14	0	0
I b	5	13	13	0	0	25	0	42	7	0	0
II	6	17	14	1	2	19	8.5	36	1	17	10
III a (Exp. 1)	6	35	30	1	4	21	20	58	1	39	10
(Exp. 2)	6	34	31	1	2	20	6	47	1	7	5
III b	6	12	12	0	0	26	0	47	13	0	0
IV	6	17	11	0	6	19	8	38	1	15	3
V	5	14	12	2	0	20	0	55	5	0	0
VI	3	7	4	0	3	11	10	22	3	20	5
VII	6	15	12	0	3	20	10	48	1	14	4
VIII	6	15	14	0	1	24	20	43	4	20	20
IX	6	20	20	0	0	22	0	44	1	0	0
X a (Exp. 1)	6	34	29	0	5	18	7	45	2	18	1
(Exp. 2)	4	21	17	1	3	18	10	40	5	14	7
X b (Exp. 1)	6	35	35	0	0	27	0	57	4	0	0
(Exp. 2)	9	28	27	1	0	26	0	58	2	0	0
(Exp. 3)	7	14	14	0	0	30	0	66	14	0	0
XI	6	40	37	0	3	26	5	68	3	10	1
Totals	114	403	364	7	32						



The diets used were very varied, and one or two were extremely poor. In order to make some sort of comparison the following table was drawn up to show the approximate amounts of fat, protein, carbohydrate and mineral salts in each diet. The figures are based on analyses given by Plimmer [1921].

Diet	Protein	Fat	Carbohydrate	Mineral salts
I <i>a</i>	11	12	71	6
II	39	8	46.5	6.5
III <i>a</i>	20	11	63	6
III <i>b</i>	21	11	63	5
IV	20	12	63	5
V	11	19	66	4
VI	8	17	66	8
VII	16.5	8.5	71	4
VIII	17	9.5	69.5	4
X	20	12	64	4

The carbohydrate and mineral salts contents of all the diets are probably within normal limits; the fat shows a wide range, but in all cases sufficient butter was given to ensure an adequate supply of vitamins A and D. Incidentally there must have been the necessary ration of vitamin E, otherwise reproduction would not have occurred. The protein of the different diets is the factor with the greatest variation, both in amount and quality. Diet VI (potato) is obviously low in protein, while diet II, with 39 %, contains an excess, since diet III *a* provides for practically maximum growth with only 20 % of the same proteins. Although diets III *a* and IV contain equal amounts of protein, the former must be regarded as a better food because it has three proteins instead of one, the protein of bread being supplemented by the proteins of milk, while in diet IV the added protein is gluten.

Taking as a criterion the rate of growth of young rats on these foods, diets VI, I *a* and I *b* are by far the worst, yet with I *a* and I *b* there was never any loss of weight during gestation. No stress can be laid on the number of litters born, because this depended entirely on the duration of the experiment. The number per litter and the weight of the young at birth varied considerably, but in no case did the experimental animals come up to the controls in either respect; moreover, with the poorer diets many of the young were born dead. It might be expected that the rat would show the greatest gain with her first pregnancy, since she breeds young and when by no means fully grown. The gain in weight at the first gestation was usually, but by no means always, large, and the gain did not decrease with each successive pregnancy. The gain in weight of the mother rat bears no relation to the size of the litter, so the eating of the placentae cannot be put forward as an explanation of the increased weight. It appears, therefore, that the characteristic sacrificing of the mother for her offspring, a noticeable feature of the lactation period, does not occur during gestation. The low weight of the young at birth, and the fact that many are frequently born dead when the mother is given a poor diet, may also be regarded as evidence in favour of this statement.

## SUMMARY.

1. Out of 403 litters born, in 364 cases the mother gained weight during gestation.
2. This gain in weight appears to be of constant occurrence and to take place when the mother is fed on a poor as well as on a good diet.

The expenses of this research were defrayed by a grant from the Medical Research Council.

## REFERENCES.

- Donaldson (1924). *The Rat*.  
Feldman (1920). *Principles of Ante-natal and Post-natal Child Physiology*.  
Greenman and Duhring (1923). *Breeding and Care of the Albino Rat for Research Purposes*.  
Hartwell (1922). *Biochem. J.* **16**, 78.  
—— (1924, 1). *Biochem. J.* **18**, 120.  
—— (1924, 2). *Biochem. J.* **18**, 1323.  
—— (1924, 3). *Lancet*, ii, 956.  
—— (1925, 1). *Biochem. J.* **19**, 729.  
—— (1925, 2). *Brit. Med. J.* i, 1073.  
—— (1926). *Biochem. J.* **20**, 751.  
—— (1927). *Biochem. J.* **21** (in press).  
Marshall (1922). *Physiology of Reproduction*.  
Plimmer (1921). *Analyses and Energy Values of Foods*.  
Smith and Chick (1926). *Biochem. J.* **20**, 131.

# LXXXI. A STUDY OF THE SYSTEM CARBONIC ACID, CARBON DIOXIDE AND WATER.

## I. DETERMINATION OF THE TRUE DISSOCIATION- CONSTANT OF CARBONIC ACID.

BY FREDERIK JOHAN JACOB BUYTENDYK, ROBERT BRINKMAN  
AND HENDRIK WILLEM MOOK.

*From the Physiological Laboratory, University of Groningen, Holland.*

*(Received March 15th, 1927.)*

ALTHOUGH the quantitative equilibrium relations in the bicarbonate-carbonic acid systems are very accurately known in biological solutions, it is a remarkable fact that only little is known about the way in which this equilibrium is attained and about the velocities of the reactions occurring. Still it might be supposed that this equilibrium is not reached at once, as in purely ionic reactions, but that it is a very definite time-process under certain conditions. Thus the buffer-capacity of a bicarbonate system, after addition of a stronger acid, is calculated from the apparent dissociation-constant ( $k_1 = 3 \times 10^{-7}$ ), but it is possible that initially the system is reacting according to the true constant of carbonic acid ( $k_1 = 4 \times 10^{-4}$ ) during a biologically significant time, with a consequent large temporary increase of the resulting  $c_H$ . If the carbonic acid were produced continuously, it might then be acting continuously as a strong acid.

On the other hand, if we consider the hydration of  $\text{CO}_2$  as a relatively slow time-reaction, the transport of this substance by the blood must be greatly influenced by its velocity of hydration, as was pointed out by Collingwood [1924].

Many questions present themselves in this way. What is the velocity of the hydration of carbonic acid *in vivo*; can  $\text{H}_2\text{CO}_3$  exist there as a strong acid for a sufficiently long time? How is the velocity of dehydration influenced by temperature,  $[\text{H}^+]$ ,  $[\text{HCO}_3^-]$ , other ions,  $\text{CO}_2$  tension, etc.? We will try first to investigate the velocity of dehydration of carbonic acid in a pure bicarbonate solution, and to estimate its true dissociation-constant. Then we will study these properties in a more physiological solution and in the circulating blood.

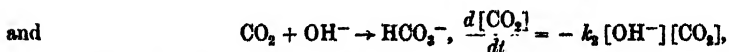
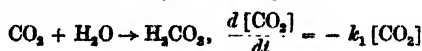
### *The hydration of $\text{CO}_2$ and dehydration of $\text{H}_2\text{CO}_3$ .*

Since the work of McBain [1912], Thiel [1913, 1916, 1922], Strohecker [1916], Thiel and Strohecker [1914, 1916], Pusch [1916] and especially of Faurholt [1922, 1924], it has been recognised that a solution of  $\text{CO}_2$  in water

contains only a small amount of the rather strong acid,  $\text{H}_2\text{CO}_3$ . If base is added to such a solution only a fraction, equivalent to the amount of  $\text{H}_2\text{CO}_3$  present, is neutralised at once; the remaining large fraction is neutralised as a time-reaction with practically the velocity of the reaction  $\text{CO}_2 + \text{OH}^- \rightarrow \text{HCO}_3^-$ . Calculation of the true dissociation-constant from the fraction of pre-existing  $\text{H}_2\text{CO}_3$  by Thiel and Strohecker, gave an approximate value of  $K_{\text{H}_2\text{CO}_3} = 4.4 \times 10^{-4}$  (in a 0.008 mol. solution of carbonic acid at  $4^\circ$ , 1.23 % is present as  $\text{H}_2\text{CO}_3$ ). The velocity of this hydration, as studied by Thiel and Strohecker, appeared to be dependent upon the  $p_{\text{H}}$  and to increase with increasing alkalinity.

Results of more detailed character have been obtained by Faurholt. His method makes use of the reaction between  $\text{CO}_2$  and amines (the carbamino-reaction), so that in a mixture of  $\text{CO}_2 + \text{H}_2\text{CO}_3$  the  $\text{HCO}_3^-$  can be precipitated as  $\text{BaCO}_3$ , while the  $\text{CO}_2$  is prevented from precipitation by addition of ammonia, methyl- or dimethyl-amine. Faurholt's conclusions are the following:

Hydration of carbon dioxide is occurring in two ways, viz.:



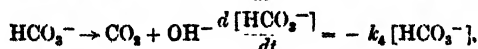
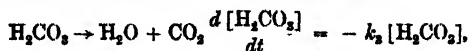
$$k_1 \text{ at } 0^\circ = 0.0013 \text{ and at } 18^\circ = 0.0011, \quad k_2 \text{ at } 0^\circ = 10^{4.61} \text{ and at } 18^\circ = 10^{4.24}.$$

Both processes are occurring simultaneously, so the hydration is determined by

$$\frac{d[\text{CO}_2]}{dt} = -(k_1 + k_2[\text{OH}^-])[\text{CO}_2],$$

which indicates that the reaction is of monomolecular type if  $[\text{OH}^-]$  is constant. If  $p_{\text{H}} < 8$ ,  $[\text{OH}^-]$  is so small that the reaction has practically the type  $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$ ; if  $p_{\text{H}} > 10$  the second reaction  $\text{CO}_2 + \text{OH}^- \rightarrow \text{HCO}_3^-$  is predominant. In the range  $p_{\text{H}}$  8-10 the velocities of both reactions are important.

The dehydration of carbonic acid is taking place in two ways:

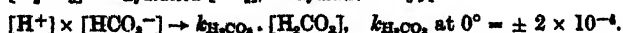


$$k_3 \text{ at } 0^\circ = \pm 1, \text{ at } 18^\circ = 7.1, \quad k_4 \text{ at } 0^\circ = 10^{-5.52}, \text{ at } 18^\circ = 10^{-4.52}.$$

Thus the dehydration of  $\text{H}_2\text{CO}_3$  is a monomolecular process; Faurholt deduces that the dehydration of the bicarbonate is also monomolecular if the  $p_{\text{H}}$  is constant. Between  $p_{\text{H}}$  8 and 10 both processes have velocities of similar magnitude, in more acid solutions  $\text{H}_2\text{CO}_3 \rightarrow \text{H}_2\text{O} + \text{CO}_2$  predominates, in alkaline solutions the dehydration of  $\text{HCO}_3^-$  prevails. In acid fluids dehydration is practically complete; between  $p_{\text{H}}$  5 and 8  $\text{CO}_2$  and carbonic acid can be present; in alkaline ranges hydration is nearly complete.

The time for 90 % establishment of equilibrium in strongly acid solutions is nearly independent of  $p_{\text{H}}$ ; at  $0^\circ$  this time is 1 sec., at  $18^\circ$   $\frac{1}{4}$  sec. At  $p_{\text{H}} = \pm 8$ , however, this time is maximal, at  $0^\circ$  about 700 sec., at  $18^\circ$  about 80 sec.; from  $p_{\text{H}}$  9 the time decreases regularly. These times have been calculated for infinite dilution and must be modified for the presence of electrolyte concentrations. Faurholt gives a probable error of 50 %.

For equilibrium conditions he finds:



Although the value of the data given by Faurholt is certainly important for biological reactions, many of them are only approximate; especially in the physiological range of  $p_H$  and temperature many questions remain unsolved. In order to make an accurate estimation of the important true dissociation-constant of carbonic acid and the types and velocities of its dehydration under physiological conditions, we have directly recorded with a lag of  $< \frac{1}{10}$  sec. the changes of  $p_H$  with an accuracy  $> 0.005 p_H$  of a bicarbonate system, after quickly mixing it with acid.

#### METHOD.

It will be clear that a method intended not only to measure but also to record  $p_H$  continuously cannot be a compensation method with zero instrument, but must be suited to record the potential differences between the unknown electrodes and constant electrodes without any appreciable polarisation. Therefore we adapted a method similar to that of Goode [1922] and followed the changes of  $p_H$  accurately and with a time lag as short as possible by connecting a suitable type of hydrogen electrode system, compensated by a potentiometer circuit, with the grid of a common triode valve and recorded the changes of the (compensated) plate current with a suitable recording galvanometer. The method is illustrated by the diagram of Fig. 1.

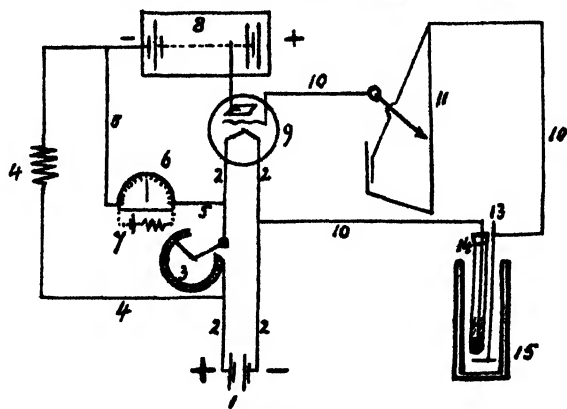


Fig. 1.

The filament of a Philips B 406 valve (9) is heated by a 4 v. accumulator (1) through the circuit (2) and the resistance (3). The plate current flows through the anode battery (8), the circuit (5) and the galvanometer (6), and can be compensated by the shunt with resistance (4). In this scheme the compensating potential is taken from the heating potential, so that a large plate potential will take too much energy from the heating cells for compensation. Therefore the maximum plate potential in this scheme can only be 30 v., and the sensitivity of the galvanometer was about 4 mm. for 1 mv. tension on the grid. This sensitivity may be increased about tenfold by removing the shunt (4), bringing the plate potential to 80 v. and compensating the plate current by a separate 2 v. accumulator, with resistance (7). In such a system it is sometimes necessary to shunt a large capacity condenser across the anode battery.

The E.M.F. of the measured system is connected between the grid and the negative pole of the heating battery (circuit 10), and can be compensated and measured by the potentiometer (11).

As experimental electrodes we used the quinhydrone system with bright Pt electrodes, the reaction time of which is as short as possible. As second electrode we used a saturated calomel electrode, or an Ag—AgCl—KCl electrode, or a narrow tube with phosphate solution (equal parts of 1/15N  $\text{KH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ), quinhydrone and a long bright Pt wire which reaches to the cotton plug by which the bicarbonate and phosphate solutions are separated. This second electrode may be cemented to the moving electrode to ensure constant distance between the two, but this proved to be unnecessary. It is important that the resistance of the electrodes is small, in order to prevent the influence of induction potentials on the measured E.M.F. If this precaution is taken and if all parts of the apparatus which can come into contact with the grid circuit have been carefully insulated, the recording of the E.M.F. of the system under study is absolutely undisturbed.

An essential part of this research was the completeness of mixing of the reacting fluids in as short a time as possible. The following method was found to be the best.

The  $\text{NaHCO}_3$  solution (100 cc. in a Dewar flask, diameter 3 cm., height 14 cm.) is stirred continuously by the Pt electrode itself which is a disc of 2 cm. diameter and moves up and down in the solution over a distance of 6 cm. twenty times a second. The connecting parts of this electrode are insulated with De Khotinsky cement, so that only the disc remains uncovered. The moving electrode is connected to the potentiometer circuit by very flexible wire and all contacts are soldered. The diluted HCl (0.95 cc.) is injected into the bicarbonate solution by means of an automatic 1 cc. syringe with strong springs; attached to the nozzle of the syringe is a straight glass tube of 0.3 cm. diameter which reaches half-way down the Dewar vessel. Through this tube the acid is injected automatically in less than 0.1 sec.

The variation in distance between the moving Pt electrode and the second constant electrode causes no deflection of the galvanometer if the resistance of these electrodes is not too high.

It might be supposed that the change of E.M.F. which follows upon the injection of HCl into the bicarbonate solution is initially caused by a temporary local concentration of free HCl at the electrode. If, however, the velocity of the mixing electrode is high enough ( $>$  ten movements per sec.) this possibility seems excluded by the following experiments:

(a) If the same amount of 0.01N HCl is injected into 100 cc. of another equimolar buffer solution (acetate-phosphate) a temporary rise of  $c_{\text{H}}$  is not noticed, but the E.M.F. is raised to its end-point in a time which is no greater than the lag of the recording system ( $< \frac{1}{25}$  sec.) (mixing time and reaction time of the quinhydrone electrode).

(b) The temporary rise in the curve indicating the  $c_{\text{H}}$  of the bicarbonate solution ( $\text{H}_2\text{CO}_3$  peak) does not occur in any  $\text{HCO}_3' - \text{H}_2\text{CO}_3$  system in which

HCl is injected, but only in those systems, in which the  $p_H$  is less than 7.5<sup>1</sup>. If this peak were caused by free HCl, we can find no reason why it should present itself only on the acid side of  $p_H$  7.5. Moreover, if one causes such a peak by injecting HCl on a slowly moving electrode, the decrease of the curve is as rapid as the increase, while the decrease of the  $H_2CO_3$  peak is much more gradual and corresponds exactly to the deduced velocity of dehydration.

*Determination of the true dissociation-constant of  $H_2CO_3$ .*

In order to measure the true dissociation-constant of  $H_2CO_3$  we recorded the  $p_H$  of 100 cc. 0.02N  $NaHCO_3$ , saturated with  $CO_2$ , into which 0.95 cc. 0.01N HCl was quickly injected and mixed (see fig. 2 and Table I). The formation of  $H_2CO_3$  causes here a rapid rise of  $[H^+]$ , which is followed at once by a slower decrease, owing to the dehydration process  $H_2CO_3 \rightarrow H_2O + CO_2$ . Our object now was to extrapolate from this registered curve of E.M.F. the initial  $c_H$  of the solution, as if no dehydration had occurred.

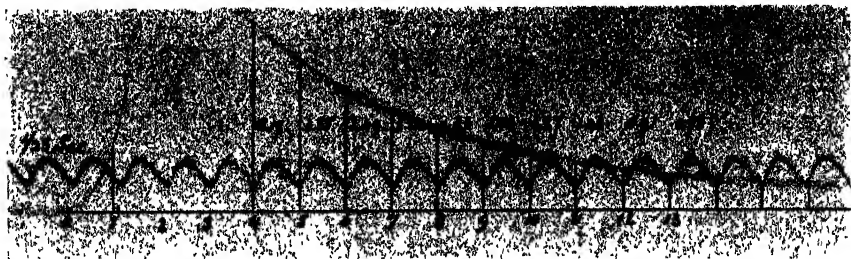


Fig. 2.

To do this, we may suppose the dehydration to be a monomolecular process at this reaction ( $p_H$  5-6), which finally reaches a dynamic equilibrium with the inverse hydration of  $CO_2$ .

Then the velocity of this reaction would be described by

$$-\frac{d[H_2CO_3]}{dt} = k_1 [H_2CO_3] - k_2 [CO_2];$$

but

$$[H_2CO_3] = \frac{[HCO_3^-] \cdot [H^+]}{K}$$

( $K$  = the true dissociation-constant of  $H_2CO_3$ ),

$$\text{therefore} \quad -\frac{[HCO_3^-]}{K} \cdot \frac{d[H^+]}{dt} = k_1 \frac{[HCO_3^-]}{K} \cdot [H^+] - k_2 [CO_2].$$

$[HCO_3^-]$  is practically constant, as the small change in its concentration caused by the dehydration of  $H_2CO_3$  may be neglected in regard to the total amount of  $NaHCO_3$  in solution.  $[CO_2]$  is also a constant as the solution is continuously kept saturated with the gas. So we have:

$$-\frac{d[H^+]}{dt} = k_1 \cdot [H^+] - k_2',$$

or by integration  $[H^+] = C \cdot e^{-k_1 t} + D$ , in which  $C$  and  $D$  are new constants; where  $D$  may be determined by the  $c_H$  at equilibrium, i.e. after infinite time.

<sup>1</sup> The influence of the reaction on the type of buffering of a bicarbonate solution will be described in the next paper of this series.

In this way we get  $[H^+] = C \cdot e^{-k_1 t} + [H^+]_{\infty}$ , or

$$\log ([H^+] - [H^+]_{\infty}) = -k_1' t + \log C \quad (k_1' = 0.434 \times k_1).$$

The experimental values of  $\log ([H^+] - [H^+]_{\infty})$  should be a linear function of the time. All curves calculated in this way showed a convexity towards the plane axis, indicating that  $k_1$  is not a constant but decreases during the reaction, which consequently cannot be monomolecular.

It was now natural to suppose that the hydrogen ions have a catalytic influence on this dehydration, as they have in many hydrations and dehydrations (formations of lactones and the inverse reactions, inversion of sucrose, etc.). Pusch [1916] made the same assumption for the hydration of  $CO_2$ .

In this case the equation becomes

$$-\frac{d[H_2CO_3]}{dt} = k_1 [H_2CO_3] [H^+] - k_2 [CO_2] \cdot [H^+];$$

taking

$$[H_2CO_3] = \frac{[HCO_3^-] \cdot [H^+]}{K}$$

( $K$  = the true dissociation-constant of  $H_2CO_3$ ),

$$\text{we get} \quad -\frac{d[H^+]}{dt} = k_1 [H^+]^2 - k_2 \cdot [CO_2] \cdot \frac{K}{[HCO_3^-]} \cdot [H^+].$$

$$\text{Let} \quad k_2 \cdot [CO_2] \cdot \frac{K}{[HCO_3^-]} = k_3,$$

$$\text{then} \quad -\frac{d[H^+]}{dt} = k_1 [H^+]^2 - k_3 [H^+].$$

By integration  $\ln \frac{k_1 [H^+] - k_3}{[H^+]} = -k_3 t + C$  in which  $C$  is an integration constant.

$$\text{If} \quad \frac{k_1}{k_3} = \alpha \text{ and } \frac{1}{[H^+]} = p \text{ then } \ln (\alpha - p) = -k_3 t + C. \quad \dots (A)$$

If  $\alpha$  is known,  $\log (\alpha - p)$  may be plotted as a function of time.  $\alpha$  was calculated in the following way.

Substituting the determined values of  $p$  for equal intervals of time, then

$$\frac{\alpha - p_n}{\alpha - p_{n+1}} = \frac{\alpha - p_{n+1}}{\alpha - p_{n+2}} = \dots = e^{-k_3(t_n - t_{n+1})} = \beta,$$

as

$$t_n - t_{n+1} = t_{n+1} - t_{n+2} = \dots$$

Thus

$$\alpha - p_n = \beta (\alpha - p_{n+1}) \text{ or } p_n = \beta p_{n+1} + \alpha (1 - \beta).$$

By taking  $p_n$  as a function of  $p_{n+1}$ , the formula must represent a straight line;  $\beta$  in this formula is the tangent of the angle with a plane axis of the constructed  $p_n/p_{n+1}$  line, which actually appears to be a straight line (Fig. 3). Thus  $\beta$  can be determined graphically;  $\alpha (1 - \beta)$  is the portion which is cut from the vertical zero line. From the graph can be seen the portion which is cut from the plane axis of  $p_{n+1} = 5.5$ ; from this portion  $\alpha (1 - \beta)$  can be determined, and as  $\beta$  is known,  $\alpha$  may be calculated.

This method of calculating  $\alpha$  has the advantage that all observations on the experimental  $p_H$  curve may be used; this would not have been possible if  $\alpha$  had been calculated from the limit-condition of the integral. The calculation of  $\alpha$  in this way and its substitution in equation (A) gives in fact a very good straight line (Fig. 4). This forms a strong argument for our supposition that hydration and dehydration of carbonic acid are proportional to the  $[H^+]$  of the system.



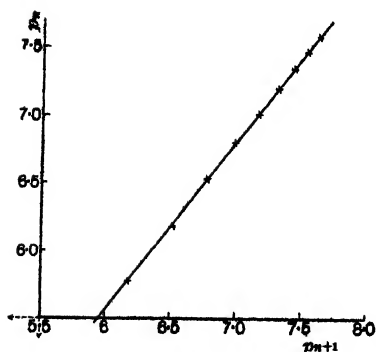


Fig. 3.

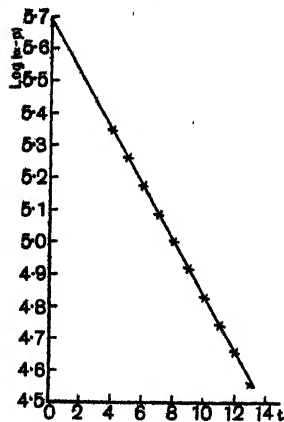


Fig. 4.

We are now able to extrapolate the  $[H^+]$  of the bicarbonate solution immediately after injection of the HCl. As an example we take the curve reproduced in Fig. 2 which had been obtained by injecting 0.95 cc. of 0.01N HCl into 100 cc. 0.02N  $NaHCO_3$  at  $14^\circ$ . The values calculated from this curve are combined in Table I.

Table I.

t.	cm.	$\Delta$ mv.	$p_H^*$	$p = \left( \frac{1}{[H^+]} \right) \cdot 10^5$	$(\alpha - p) \cdot 10^5$	$\log(\alpha - p)$
4	4.17	8.45	5.762	5.78	2.22	5.346
5	3.35	6.79	5.791	6.18	1.82	5.260
6	2.69	5.45	5.814	6.52	1.48	5.170
7	2.20	4.46	5.832	6.79	1.21	5.083
8	1.83	3.71	5.845	7.00	1.00	5.000
9	1.53	3.10	5.856	7.18	0.82	4.914
10	1.27	2.57	5.865	7.33	0.67	4.826
11	1.06	2.15	5.872	7.45	0.55	4.740
12	0.91	1.84	5.878	7.55	0.45	4.653
13	0.76	1.54	5.883	7.64	0.36	4.556

Calibration of the curve 10 mv. = 4.94 cm.

Initial  $p_H$  before addition of HCl = 5.91.

Graphical determination of  $\beta = 1.221$ .

Graphical determination of  $\alpha = 8.00 \times 10^5$ .

$k_2 = 2.16$  (Brigg. log., time in sec.)†.

So  $k_1 = \alpha \times k_2 = 8.00 \times 10^5 \times 2.16 = 1.73 \times 10^6$ .

Found by extrapolation  $\log(\alpha - p_0) = 5.68$  (see Fig. 4).

$p_0 = 3.16 \times 10^5$ .

$H_0 = 3.16 \times 10^{-6}$ .

\* The absolute strength of these  $p_H$  values has not been determined in three decimals, but the relative differences in  $p_H$ , which are the only important values for the determination of  $\alpha$  and  $\beta$ , may be given in three decimals.

†  $k_2$  is, according to equation (A), equal to the tangent of the angle which the straight line of Fig. 4 makes with the plane axis.

The true dissociation-constant  $K$  was calculated as follows.

Concentration of H before injection of HCl	= $H_1$
" " H immediately after the injection	= $H_0$
" " $H_2CO_3$ before injection	= $c_1$
" " $HCO_3^-$ before injection	= $c_2$
" " HCl (diluted to 100 cc.)	= $c_3$

After the injection of HCl the concentration of  $\text{H}_2\text{CO}_3$  must be  $c_1 + c_3$  and of  $\text{HCO}_3^- = c_2 - c_3$ .

Before injection we have  $c_1 \times K = H_1 \times c_2$ ,  
and after  $(c_1 + c_3) \times K = H_0 \times (c_2 - c_3)$ ,

$$\text{so } K = \frac{H_0 \times (c_2 - c_3) - H_1 \times c_2}{c_3}.$$

In our example we had  $H_1 = 1.23 \times 10^{-6}$ ,  $H_0 = 3.16 \times 10^{-6}$ ,  $c_2 = 2 \times 10^{-2}$ ,  $c_3 = 9.5 \times 10^{-5}$ , so  $K$  at  $14^\circ = 4.0 \times 10^{-6}$ .

We have made twelve similar experiments at temperatures between  $14^\circ$  and  $18^\circ$  and always found values between  $K = 1.5$  and  $4 \times 10^{-6}$ . Researches in which the constancy of temperature is considered, and determinations at  $37^\circ$  are in progress.

The graphical determination of  $\beta$  gave a  $k_3$  of 2.16; as  $\alpha = \frac{k_1}{k_3}$  we find  
 $k_1 = 8.00 \times 10^{-5} \times 2.16 = 1.73 \times 10^{-6}$ .

Thus at  $p_H = 6$ , the dehydration constant of  $\text{H}_2\text{CO}_3 = 1.73$ , which indicates that the time for semi-completion of the dehydration is 0.17 sec. at  $14^\circ$ .

If it is permissible to extrapolate this value for a physiological  $p_H$  of 7.35, we get for semi-completion about 4 sec. So it seems quite probable that the existence of  $\text{H}_2\text{CO}_3$  in a bicarbonate solution may occur for a biologically important time.

Hydration of  $\text{CO}_2$  at  $p_H$  7 seems to be a very slow reaction (cf. Collingwood [1924]).

The relation of the constants of hydration and dehydration is given by the equilibrium constant of  $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$ :

$$\frac{k_{\text{hydration}}}{k_{\text{dehydration}}} = K_{\text{equilibrium}} = \frac{[\text{H}_2\text{CO}_3]}{[\text{CO}_2]} = (\text{approx.}) \frac{\text{apparent diss.-const. of carbonic acid}}{\text{true diss.-const. of carbonic acid}} \\ = \frac{3 \cdot 10^{-7}}{4 \cdot 10^{-4}} = 7.5 \times 10^{-4}.$$

Another way of calculating the equilibrium constant is the following. According to Strohecker there is in a certain solution of carbon dioxide 99.44 % present in the form of  $\text{CO}_2$  and 0.05 % as undissociated  $\text{H}_2\text{CO}_3$ ; this gives for the equilibrium constant  $\frac{0.05}{99.44} = 5 \times 10^{-4}$ , which agrees fairly well with the calculation from the true and apparent dissociation-constant, but not with Faurholt's value. If we calculate the hydration constant of  $\text{CO}_2$  from the relation

$$\text{hydration constant} = 7.5 \times 10^{-4} \times \text{dehydration constant},$$

we get at  $p_H$  7.35

$$k_{\text{hydration}} = 7.5 \times 10^{-4} \cdot k_{\text{dehydration}} = 5.85 \times 10^{-5},$$

and the time for semi-completion of hydration is  $\frac{0.301}{5.85 \times 10^{-5}} = 5.2 \times 10^3$  sec.

or about  $1\frac{1}{2}$  hours. Moreover, the inverse reaction is retarding hydration, but it might be possible that the reaction  $\text{CO}_2 + \text{OH}^- \rightarrow \text{HCO}_3^-$  is coming into play at this  $p_H$ .

The true dissociation-constant of  $\text{H}_2\text{CO}_3$  being  $4 \times 10^{-4}$ , we may now estimate the buffer value of the bicarbonate solution, immediately after the addition of the acid.

Van Slyke's formula for molar buffering capacity is

$$\frac{dB}{cdp_H} = 2.3 \frac{k \cdot [H]}{(k + [H])^2}.$$

So the buffering capacity of the bicarbonate system in final equilibrium at  $p_H$  7 is

$$2.3 \frac{3 \cdot 10^{-7} \cdot 10^{-7}}{16 \cdot 10^{-14}} = 0.43.$$

Immediately after addition of an acid, when the system is reacting according to its true constant, we get  $2.3 \frac{4 \cdot 10^{-4} \cdot 10^{-7}}{(4 \cdot 10^{-4} + 10^{-7})^2} = 5.8 \times 10^{-3}$ , a very small buffer value.

Also the second dissociation-constant ( $6 \times 10^{-11}$ ) gives a very small buffering capacity, viz.  $13.8 \times 10^{-4}$ . It must be concluded, therefore, that immediately after addition of an acid the bicarbonate system cannot exert buffering action at  $p_H$  7.

Preliminary experiments with serum and blood indicate that the presence of free  $\text{H}_2\text{CO}_3$  after addition of  $\text{HCl}$  cannot be demonstrated at all. Also there is quite another cause of changes occurring before attainment of equilibrium when  $\text{HCl}$  is added to ultrafiltrate. These problems will be dealt with in the next paper of this series.

#### SUMMARY.

1. The change in  $[H^+]$  in a bicarbonate solution to which strong acid was added was studied by means of continuous observation of the  $p_H$  of the system with an accuracy of 0.005  $p_H$  and a time lag not exceeding  $\frac{1}{15}$  sec.
2. The true dissociation-constant of  $\text{H}_2\text{CO}_3$  and the hydration and dehydration velocities were determined; they appeared to have biologically important values.
3. The velocity of dehydration was shown to be proportional to the  $[H^+]$  of the system.
4. At  $p_H$  about 7 the bicarbonate system has practically no immediate buffering capacity.

#### REFERENCES.

- Collingwood (1924). *J. Physiol.* 59, 22.  
 Faurholt (1922). *Z. allgem. Chem.* 120, 85.  
 — (1924). Dissertation (Copenhagen).  
 Goode (1922). *J. Amer. Chem. Soc.* 44, 26.  
 McBain (1912). *J. Chem. Soc.* 101, 814.  
 Pusch (1916). *Z. Electrochem.* 22, 206, 293.  
 Strohecker (1916). Beitr. z. Kenntniss der Wass. Lös. der Kohlensäure. Dissertation (Marburg).  
 Thiel (1913). *Ber. deutsch. chem. Ges.* 46, 231, 867.  
 — (1916). *Z. Electrochem.* 22, 423.  
 — (1922). *Z. allgem. Chem.* 121, 211.  
 Thiel and Strohecker (1914). *Ber. deutsch. chem. Ges.* 47, 945, 1061.  
 — (1916). *Z. Unters. Nahr. Genussm.* 21, 121.

## LXXXII. ON THE DEVELOPMENT OF CHROMOGENIC PROPERTIES IN CHOLESTEROL BY THE ACTION OF HEAT.

BY THOMAS MOORE AND STANLEY GORDON WILLIMOTT.

*From the Biochemical Laboratory, Cambridge.*

*(Received April 29th, 1927.)*

"OXYCHOLESTEROL," a brown resinous substance first obtained by Lifschütz [1908] from cholesterol by the action of benzoyl peroxide in acetic acid solution, came into prominence through certain similarities shown by its colour reactions to those associated with vitamin A [Takahashi *et al.*, 1924, 1925]. More recently Rosenheim [1927], working on the arsenious chloride reaction, has also obtained a chromogenic substance from cholesterol by the use of the same reagent, but in chloroform solution, which is found to simulate the "vitamin" colour reactions closely in many respects, though proving unstable in the presence of oils. It is intimated that further experiments are being undertaken using ergosterol, the precursor of vitamin D, in place of cholesterol.

In the meantime certain experiments have come to our notice in which "oxycholesterol" has been formed from cholesterol by other means than the use of benzoyl peroxide or a similar oxidising agent.

(1) It will be recalled that Rosenheim and Webster [1926] suggested that "oxycholesterol" might be formed on the irradiation of cholesterol in air, thus increasing the non-precipitable fraction without raising the antirachitic value. Shear and Kramer [1926] have now confirmed the appearance of this substance under such conditions, having obtained by the prolonged irradiation of cholesterol in air a yellow amorphous product, "U.V. Oil," which gives the typical colour reaction of "oxycholesterol" as described by Lifschütz.

(2) In the course of experiments on the multiplication of infusoria, Robertson [1925], by the aeration of an aqueous colloidal solution of cholesterol at boiling-point in the presence of traces of an acetone extract of brain, has obtained on evaporation a "light brown pasty mass," resembling "oxycholesterol." This material is found to give the typical colour reaction, and is supposed to be produced from the sterol by the action of a catalyst present in the brain extract, in the absence of which no "oxidation" takes place.

In the course of experiments designed to confirm such observations we were led to examine the colour reactions of cholesterol which had been gently heated, both in aqueous colloidal solution and in the solid state. The results stated below would indicate that caution is necessary in accepting the production of "oxycholesterol" as a specific effect of some particular mechanism in cases where heat is also involved.

*Experimental.*

The experiments were first carried out on a specimen of cholesterol obtained by five recrystallisations from a crude cod-liver oil product supplied by Messrs Joseph Nathan, Ltd. They were repeated on an "almost pure" sample, m.p. 148°, from the same source, obtained by Prof. Heilbron in the course of his recent spectroscopic work, and almost free from ergosterol. Both specimens gave negative results when tested with (1) a 1-5 sulphuric-acetic acid mixture, followed by a drop of 2 % ferric chloride in acetic acid (Lifschütz), or (2) a chloroform solution of antimony trichloride [Carr and Price, 1926], which were the reagents employed to test for "oxycholesterol."

*The production of "oxycholesterol" in molten cholesterol.*

Samples of cholesterol were heated to temperatures slightly above the melting-point in an open dish on a carefully regulated sand-bath. After some time a slight yellow tint was observed, and on cooling the cholesterol was now found to give positive reactions with the reagents mentioned above, the nature of such reactions depending upon the duration of heating and the temperature attained.

(a) If the cholesterol were warmed for a few minutes at a temperature barely above the melting-point a very faint yellow tint was acquired. With the antimony trichloride reagent a blue coloration was given, which faded in a few minutes to a dull brown. With the Lifschütz reagents a preliminary violet was given, which became ultramarine and then greenish blue, but not emerald green, on the addition of ferric chloride. (b) If the cholesterol were kept at the same temperature for a longer period (30-120 mins.) the yellow tint developed was more pronounced. A bright ultramarine, permanent for hours, was now given with the antimony trichloride reagent, while in the Lifschütz test a definite green was obtained on the addition of ferric chloride. (c) Prolonged heating at more elevated temperatures converted the cholesterol entirely into brown vitreous resins. Some resins gave the complete "oxycholesterol" colour sequence with the Lifschütz reagents, and with antimony trichloride an indigo blue coloration; other resins, in the production of which still higher temperatures were attained, gave no colorations other than brown.

It would seem, therefore, that the production of "oxycholesterol" effected by Shear and Kramer through the agency of ultra-violet radiations, may equally well be effected by thermal means. These workers have also found that irradiated cholesterol gives on boiling with a mixture of 15 parts aniline and 1 part hydrochloric acid a red or reddish brown coloration, which, in spite of strong criticism by Rosenheim and Webster, is still attributed to vitamin D. It may be stated, in support of the view of Rosenheim and Webster, that samples of melted cholesterol were found to react positively to this test, though the original cholesterol gave no coloration. Moreover, samples of a cholesterol,

freed from ergosterol by bromination and reduction, after melting gave positive reactions in this test and in those for "oxycholesterol." The aniline hydrochloride test cannot be considered specific to vitamin D.

*The "oxidation" of cholesterol in colloidal solution.*

In confirmation of Robertson's work it was found that stable colloidal solutions of cholesterol could be obtained by pouring an alcoholic solution of the latter into hot water, with constant agitation. In our experiments 5 cc. of a 0.5 % solution were poured into 100 cc. of hot distilled water, 105 cc. of solution therefore contained 25 mg. of cholesterol. The solutions so obtained displayed a slight orange opalescence in transmitted light and remained stable for days. As the solutions were not to be used in physiological experiments it was thought unnecessary to follow Robertson's procedure for the removal of alcohol and the solutions were used without further treatment.

After aeration of the colloidal solutions under a reflux condenser at 90–100° for 6 hours with traces of acetone extracts of brain, liver, or kidney, the residues after evaporation gave erratic results when tested with the Lifschütz "oxycholesterol" reagents, the initial colours obtained varying from bright purple to dull brown, which was at first mistaken for a negative reaction. It soon became apparent, however, that the colour produced was entirely dependent on the care taken during evaporation. Cautious evaporation resulted in the production of the normal purple of "oxycholesterol," even in the absence of tissue extracts; overheating in the final stages resulted in a brown colour reaction. Moreover, plain solutions which had not been aerated for 6 hours but merely evaporated carefully to dryness gave positive tests for "oxycholesterol." For a further investigation of the phenomenon it was necessary to evaporate the solutions at a lower temperature. Accordingly they were poured after aeration into large Petri dishes, and left in a hot room at 37° until dry.

Immediate colorations with the Lifschütz and antimony trichloride reagents were given by residues so obtained from solutions heated, without tissue extracts, to 90° for periods down to 1 hour, with or without aeration. No colorations were produced by residues from (a) a solution made up and aerated for 48 hours at 37°, (b) a solution made up hot and immediately cooled, and (c) an alcoholic solution of cholesterol evaporated directly.

A further phenomenon is perhaps worthy of mention. The colloidal cholesterol solutions immediately after preparation were very sensitive to the precipitating action of electrolytes; after heating for some hours they became much less sensitive. Such increased stability would seem to coincide with the formation of the resinous "oxidation" products, but the question has not been investigated in detail.

#### DISCUSSION.

Cholesterol has long been known to undergo changes on heating. The above experiments show that chromogenic properties appear on such treatment, and consequently in all experiments where the presence or production

of "oxycholesterol" is observed care must be taken to rule out such thermal effects as might arise in the course of the experiment or during the processes of isolation. Thus Lifschütz [1914] has described experiments in which "oxycholesterol" is produced by heating defatted blood with cholesterol in acetic acid solution at 60–65° for several days. While it is possible that the blood acts as a catalyst under these conditions, such evidence does not, in our opinion, justify the assumption of a similar catalytic action in normal physiological processes.

In regard to the experiments of Robertson, as stated above, we have been unable to confirm the reported stability of cholesterol towards "oxidation" when aerated in colloidal solution in the absence of brain extract. Since it is stated that the cholesterol to resist oxidation must be "perfectly pure," we took pains to repeat our experiments on as pure a specimen of cholesterol as was available to us, but still obtained the "oxycholesterol" colour reactions on evaporation. In the absence of a more detailed description of Robertson's experiments we are at a loss to explain this discrepancy.

Cholesterol, of course, may be boiled in most organic solvents without the formation of "oxycholesterol," and the processes of crystallisation and evaporation from such solvents only present danger when excessive temperatures are attained. Assuming that due precautions were taken against this in the experiments of Shear and Kramer all that can be said is that the changes produced in cholesterol by heating and by irradiation in air are superficially very similar.

In conclusion, it should be noted that in our own experiments the cholesterol was usually only in part converted into resinous products, and in some cases only a fraction of such resins must have possessed chromogenic properties. Heat was usually much less efficient in the production of chromogenic substances than the benzoyl peroxide procedure described by Lifschütz.

#### SUMMARY.

Brown resinous substances are produced in cholesterol on melting or on heating in aqueous colloidal solution. At the same time chromogenic properties similar to those of "oxycholesterol" are acquired.

Our thanks are due to Sir F. G. Hopkins for support and criticism, also to Prof. Heilbron for samples of cholesterol.

#### REFERENCES.

- Carr and Price (1926). *Biochem. J.* **20**, 497.  
 Lifschütz (1908). *Ber. deutsch. chem. Ges.* **41**, 252.  
 ——— (1914). *Z. physiol. Chem.* **93**, 209.  
 Robertson (1925). *Austral. J. Exp. Biol. Med. Sci.* **2**, 83.  
 Rosenheim (1927). *Chem. Ind.* **46**, 269.  
 Rosenheim and Webster (1926). *Biochem. J.* **20**, 544.  
 Shear and Kramer (1926). *J. Biol. Chem.* **71**, 213.  
 Takahashi, Nakamiya, Kawakami and Kitasato (1924). *Bull. Inst. Phys. Chem. Research*, **3**, 693.  
 ——— (1925). *Scientific Papers Inst. Phys. Chem. Research, Tokyo*, **3**, 81.

# LXXXIII. THE $p_H$ OF THE GASTRO-INTESTINAL TRACT OF CERTAIN RODENTS USED IN FEEDING EXPERIMENTS, AND ITS POSSIBLE SIGNIFICANCE IN RICKETS.

By THIRZA REDMAN, STANLEY GORDON WILLIMOTT  
AND FRANK WOKES

*From the Biochemical Laboratory, Cambridge, and the University of Liverpool.*

*(Received May 2nd, 1927.)*

ALTHOUGH determinations of the  $p_H$  of the contents of different parts of the gastro-intestinal tract have been carried out by many previous investigators on such widely different animals as human beings, dogs, cats, pigs, cows and sheep, very little attention appears to have been paid to the rodents so largely used in feeding tests for vitamins. This may be due in part to the greater difficulties involved in handling the minute quantities of material available in such small animals as guinea-pigs, rats and mice. But, in view of the fact that vitamins have already been shown in a number of cases to be affected by the reaction of the medium in which they are present, it seemed to us worth while to investigate the intestinal conditions in these particular rodents.

In the case of the albino rat and vitamin D, interest has been aroused by results recently published by several workers. Zucker and Matzner [1924], working on the faeces only, found that when rats were fed on a rachitogenic diet (Sherman and Pappenheimer's No. 84) the faeces became alkaline ( $p_H$  7.4–8.0), and that administration of vitamin D, either in the form of cod-liver oil, or by direct irradiation of the animals, was followed by a change, within a few days, to  $p_H$  6.0–6.4, although the rachitogenic diet was still continued. After administration of a vitamin D-free cotton-seed oil, this change in faecal reaction from alkalinity to acidity did not take place. These interesting results obtained by Zucker were confirmed by Jephcott and Bacharach [1926], who found that the change followed also after either direct irradiation of the animals or administration of irradiated cholesterol or of the vitamin D fraction of cod-liver oil (ostelin). The faeces, however, represent merely the end of the story. The absorption of calcium and phosphorus, which is closely connected with the rôle of vitamin D, probably takes place mainly in parts of the gastro-intestinal tract where the faecal condition has not yet been reached. It is, therefore, an obvious duty to compare the change in faecal reaction with any



alterations which may simultaneously occur in the  $p_H$  of the entire gastrointestinal tract both on deprivation of vitamin D, and on its readministration to rachitic animals.

An examination of the methods used by other workers indicated some of the obstacles to be expected. It was necessary to collect the material with all precautions possible in order to avoid contamination or undue exposure, and to examine it as soon as possible after the death of the animal. Diet, age, sex, time after last meal, and state of health of the experimental animal were amongst the factors to be considered. Whilst we have attempted to overcome all these difficulties, and numerous others which arose in the course of the work, we realise that the possibility still remains of some unknown factor having been overlooked, and would place this reservation on the results here recorded.

#### EXPERIMENTAL.

##### *Collection of material.*

The animals were killed by chloroform. The abdomen was opened immediately, and the stomach ligatured at both ends. The oesophagus was cut above the ligature, and the large intestine just above the anus. The whole of the gastrointestinal tract was then removed and measured. Ligatures were applied at each end of the various sections of intestine whose  $p_H$  was to be determined. The stomach was rapidly washed with cold water, opened, and the whole of the contents removed and well mixed. A portion was quickly stirred with sufficient distilled water to make a liquid which could be poured into the electrode vessel; a small quantity was set aside for colorimetric estimation, and the remainder triturated with quinhydrone, poured into the electrode vessel, and the  $p_H$  determined as described below. The same procedure was adopted with each section of the tract examined, the principle being to avoid contamination by careful washing of the outside of the ligatured section, and to minimise exposure to the air by doing one section at a time, and transferring the mixed material to the closed electrode vessel as quickly as possible. In dealing with the material from the large intestine, when this was of too firm a consistency to mix readily by stirring, it was quickly broken down by rubbing on a glazed porcelain tile with a stiff spatula and just sufficient distilled water to make a thick liquid paste.

##### *Colorimetric estimation.*

Colorimetric estimations of the  $p_H$  of gastric and duodenal contents have been made by many workers, the methods adopted usually being modifications of Henderson and Palmer's [1912] original technique for estimating the  $p_H$  of urine, by comparing the colour given by the material or an aqueous extract on addition of a suitable indicator with the colour given to the same indicator by a standard buffer solution of known  $p_H$ . In the case of urine,

which is a fairly stable and well buffered fluid, it is moderately easy to obtain duplicate results agreeing to within 0.1  $p_H$ . When dealing with the contents of the stomach and intestines, however, a number of difficulties arise. In the first place, as has been shown by Kahn and Stokes [1926] in the case of the stomach, and by our own results throughout the gastro-intestinal tract, the material is not usually noticeably buffered until the ileum is reached. Since only minute amounts of material are available from the smaller animals, we were unable to apply Henderson and Palmer's method, which requires the use of a comparator, on account of the risk of dilution. Secondly, as Kahn and Stokes also showed, the most serious difference between colorimetric and electrometric results on gastric contents is due to loss of carbon dioxide, especially in faintly acid or alkaline solutions, and clearing the solution by centrifuging or filtering made very little improvement. Thirdly, there are the well-known protein and salt errors, which may be responsible for differences of 0.2 to 0.5  $p_H$ , but are less serious than the loss of carbon dioxide. On the whole, these sources of error appear to diminish as the material passes down the gastro-intestinal tract, because we usually found widest divergence between colorimetric and electrometric results in the stomach, duodenum and jejunum, less in the ileum, and fairly close agreement in the caecum and large intestine. Our method of colorimetric estimation was as follows. About 0.05 g. of the fresh material was taken up on a standard platinum loop, and mixed on a white glazed porcelain tile with about 0.1 cc. of diluted indicator. If the material was too viscous, it was diluted with two or three times the quantity of distilled water immediately before taking the reading. The colour obtained was compared at once with those given by similar loopfuls of standard buffer solutions of known  $p_H$ , mixed each with 1 drop of diluted indicator. The indicators used were thymol blue, bromophenol blue, methyl red, bromocresol purple, bromothymol blue, phenol red and cresol red, as recommended by Clark [1920], but further diluted to about 0.005 %. In every case a change of colour was used, and wherever possible readings were taken on the same sample with two or three different indicators. Duplicate results usually agreed to 0.2  $p_H$ . The method requires the minimum amount of material and of dilution, and involves only a short time of exposure and loss of carbon dioxide. It gave the most satisfactory results in the caecum and large intestine, where the difference between colorimetric results in over a hundred samples was never more than 0.4  $p_H$ , and averaged less than 0.2.

#### *Electrometric estimation.*

Electrometric estimations of the  $p_H$  in various parts of the small intestine have been carried out by several previous investigators on larger animals. McClendon [1915] and his co-workers, in their numerous experiments on men, cats and dogs, appear to have used generally a special type of hydrogen electrode, and quote results to 0.1  $p_H$ . Kahn and Stokes [1926], using a hydrogen electrode to measure the human gastric  $p_H$ , claim to have secured an

accuracy of 0.05  $p_H$ . Long and Fenger [1917], working on material from the stomach and duodenum of men, and the jejunum and ileum of pigs, calves, lambs and a rabbit, and using a hydrogen electrode in a Hasselbalch vessel, quote  $p_H$  results to the second decimal place, but do not state what was their experience of the various possible sources of error. We have not been able to discover records of any  $p_H$  estimations on the caecum and large intestine. A great deal of work has been done on the faecal  $p_H$  in men and different animals. Robinson [1925] compared the results obtained with the hydrogen electrode and the quinhydrone electrode on a series of samples of faeces whose  $p_H$  ranged from 5.0 to 7.8, and found the difference to be 0.0 to 0.3, with an average of less than 0.1.

The quinhydrone electrode, since its introduction by Büllmann [1921], has been employed by a number of investigators, and found to give reliable results within the  $p_H$  range of 1 to 8. The greatest advantages of the quinhydrone electrode over the hydrogen electrode for this particular work were:

(1) the rapidity and ease with which equilibrium is maintained, as compared with the time required, and difficulty of making readings, when using the hydrogen electrode;

(2) the consequent small loss of carbon dioxide, especially as a current of hydrogen is avoided, and a closed vessel can be used. Three types of electrode vessels were used, one designed by Corran [Corran and Lewis, 1924], and having a gold electrode, and two capillary vessels similar to those recently described by Cullen and Büllmann [1925] in which we used gold-plated platinum electrodes. The formula from which results were calculated was the following:

$$p_H = \frac{0.9802 - (0.5600 + E)}{0.058}$$

$E$  being the observed E.M.F. of the following cell:

Hg/Hg<sub>2</sub>Cl<sub>2</sub>/M KCl/Saturated KCl/Material saturated with quinhydrone/Au.

After making numerous parallel observations on different samples, we are of the opinion that our average experimental error with the quinhydrone electrode, which was used throughout this work, was less than 0.1  $p_H$ . Although in some of our tables the electrometric results are given to two places of decimals in order to show the variations obtained, we do not consider the second place significant. As will be seen later, a variation from the true  $p_H$  of less than 0.1 would not have any important effect on the conclusions to be drawn from our experimental work. When using the capillary electrode vessels, especially the straight tube type requiring only a few drops of liquid, diffusion was minimised by taking the reading as rapidly as possible, as suggested by Büllmann and Lund [1921].

### *Experiments on guinea-pigs.*

Our first experiments were carried out on guinea-pigs, as it was thought advisable to gain experience on moderate-sized animals before turning to the

smaller rats on which the bulk of the work was to be done. The results, however, may be of interest in connection with problems lying outside our own field of work, such as, for instance, the possible relationship between scurvy and acidosis [Lepper and Zilva, 1925].

The guinea-pigs used were normal animals, reared in the Bacteriological Department of the Liverpool City Laboratories. They were fed on a diet of bran, oats, cabbage and water *ad lib.*, and were all in good health. A number of these animals were chloroformed at different times after their daily meal, the gastro-intestinal tract was removed as above described, and the  $p_H$  of its contents determined, colorimetrically and electrometrically, at various points throughout its course. It was found that the colorimetric and electrometric results agreed fairly closely (to within 0.2 or 0.3  $p_H$ ) when applied to the contents of the ileum, caecum and large intestine, but showed a wide divergence in the duodenum and jejunum, the colorimetric readings always being 0.3 to 1.5  $p_H$  higher than the corresponding electrometric readings. The colorimetric readings on the gastric contents were also higher than the electrometric, but the difference was not so great (0.2–1.0  $p_H$ , average 0.5). This is in agreement with the results obtained by Kahn and Stokes [1926], working on human gastric material.

Table I. *Comparison of results obtained by colorimetric and by electrometric methods for  $p_H$  of contents of gastro-intestinal tract of a normal guinea-pig.*

Animal ♂, 6 months old, killed 4 hours after meal.

	$p_H$	
	Colorimetric	Electrometric
Stomach	4.2–4.4	3.83
Duodenum	6.2–6.5	5.04
Small intestine—		
10–20 cm. below duodenum	6.6–6.8	6.23
30–40     "     "	7.2–7.6	7.70
50–60     "     "	7.3–7.6	7.80
80–90     "     "	7.2–7.4	7.57
Caecum	6.0–6.2	6.37
Large intestine—		
5–10 cm. below caecum	6.2–6.5	6.26
25–30     "     "	6.0–6.2	6.26
35–40     "     "	5.6–5.9	5.74
45–50     "     "	5.6–5.8	5.80
55–60     "     "	5.6–6.0	5.80

In Table I are given details of a typical example, in which estimations were made at as many points as possible. The figures given for the colorimetric results represent the extreme limits reached when each sample was tested with at least two different indicators, and were practically always obtained by independent observers unaware of the electrometric result. The electrometric readings were taken at first in a small electrode vessel whose capacity was about 2 cc. In order to obtain this quantity from the amount of intestinal material available, it was necessary to dilute the latter five to ten times. The

question immediately arose as to what effect this would have on the  $p_H$ . An experiment was therefore performed in which longer lengths of the intestine were taken, and sufficient of the contents obtained to make up a series of dilutions at each point. The results are shown in Table II, and from this and a number of similar experiments it would appear that the error due to dilution is greatest in the stomach, duodenum and ileum, and that the gastro-intestinal contents become more effectively buffered as one passes down the tract. On this account we decided to reduce the capacity of our electrode vessel, and after various trials we finally adopted the two types of capillary electrodes previously described, which enabled us to work with 1 in 3 or 1 in 2 dilutions. Judging from a large number of observations on this material in widely different dilutions, including some made with the Kerridge [1925] glass electrode, which permits the use of practically undiluted material, we are of the opinion that our electrometric results obtained with approximately 1 in 3 dilutions do not vary more than 0.1 to 0.2 from the actual  $p_H$  of these parts. In the caecum and large intestine the error is probably smaller. (The effect of dilution on the  $p_H$ , determined colorimetrically and electrometrically, of faintly alkaline buffered solutions containing varying amounts of sodium chloride has been studied by Lepper and Martin [1926] who found changes of 0.05 to 0.2  $p_H$  when diluting four times.)

We next investigated the effect of chloroforming the animals at different times after the daily meal. Six guinea-pigs were examined, the period varying from  $1\frac{1}{2}$  to 17 hours, and the results obtained are summarised in Fig. 1. In plotting these results, the total length of the tract from stomach to anus was taken as a unit, and the various sections of the tract were expressed as fractions of that unit. This enabled an accurate comparison to be made of results obtained on animals of different sizes.

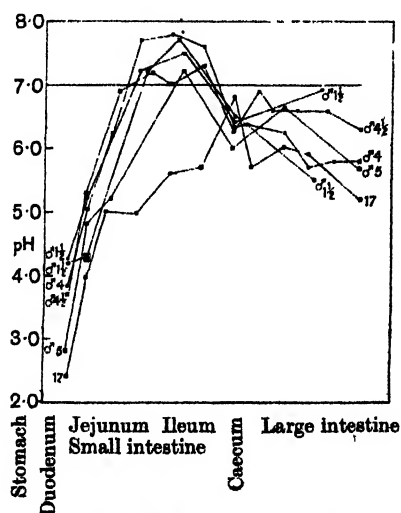


Fig. 1.  $p_H$  of gastro-intestinal tract of young normal guinea-pigs.

Table II. *Effect of dilution on the  $p_H$  of the contents of the gastro-intestinal tract of a normal guinea-pig.*

Animal ♂, 7 months old, killed  $1\frac{1}{2}$  hours after meal.

	$p_H$			
	Colorimetric	Electrometric		
Dilution	1/3	1/5	1/50	1/250
Stomach	4.8-5.2	4.26	5.04	—
Duodenum	5.8-6.2	5.22	6.62	6.52
Small intestine—				
20-50 cm. below duodenum	6.8-7.2	7.22	6.69	6.61
50-80 " "	7.4-7.8	7.48	6.93	6.73
Caecum	6.2-6.4	6.41	6.52	6.62
Large intestine—				
30-35 cm. below caecum	6.8-7.0	6.93	6.62	6.52

In general, it was found that the acidity of the contents of the stomach and duodenum tends gradually to increase during the first 4 hours after ingestion of a meal, which is in agreement with the findings of McClendon [1915] on human subjects. In the remainder of the tract, however, no appreciable difference could be observed in the  $p_H$  results obtained when the time between feeding and death was anything between  $1\frac{1}{2}$  and  $4\frac{1}{2}$  hours. Only when food was withheld for as long as 17 hours was there a markedly greater acidity throughout the small intestine. Omitting this abnormal case, the results obtained on the other five animals may be summarised as follows: stomach  $p_H$  2.8-4.3, average 3.8; duodenum 4.8-5.3, average 5.1; jejunum 5.1-7.1; ileum 7.0-7.8, average 7.4; caecum and large intestine 5.2-6.9, average about 6.4. The stomach was always strongly acid, there was a decrease in acidity in the duodenum and ileum, so that the small intestine was mainly alkaline, and the caecum and large intestine, on the other hand, were always acid.

Another point investigated was the effect of entry of bile during dissection. In one animal the common duct was ligatured immediately after opening, and the duodenal contents were found to be more acid ( $p_H$  4.3) than in any of the other four animals. On adding to the duodenal contents 1 cc. of an aqueous extract (about 5%) of the gall bladder, the  $p_H$  was raised to 5.3, the usual value in unligatured animals. These results indicated that the observed  $p_H$  of the gastro-intestinal tract might be appreciably affected either by the time of killing, or by the method of dissecting, the experimental animal. The method of killing usually adopted was by chloroforming, but in one or two cases where a blow on the head had been employed no appreciable difference was observed in the  $p_H$  results obtained.

#### *Experiments on rats.*

Having gained in the experiments on guinea-pigs some knowledge of the special difficulties involved, we next turned our attention to rats, on which the more important work was to be done. Both albinos (inbred from Wistar Institute ancestors) and piebalds were used, and gave similar results. The same technique was employed in preparing the experimental material, and in

taking the colorimetric and electrometric readings. A discrepancy was again observed between the colorimetric and the electrometric readings in the duodenum and ileum. Undue dilution was avoided by the use of the capillary electrodes. The accuracy of these electrodes was tested by taking a number of parallel readings on material from different animals, and it was found that they gave results agreeing to 0.2  $p_{\text{H}}$  or less, the average difference being below 0.1, as shown in Table III.

It was not possible to test the effect of cutting off the supply of bile, because the rat does not possess a gall bladder [Donaldson, 1924], and we were also unable to find the common duct. Whether this hitherto little recognised fact is associated with differences in the digestive processes is a problem for further investigation.

Table III. *Comparison of results obtained with the two types of capillary electrodes, on normal rats.*

Animal Electrode	Albino (12 months old)		Piebald (2 months old)	
	Straight	Side-tube	Straight	Side-tube
	$p_{\text{H}}$			
Stomach	3.04	3.02	3.97	3.93
Duodenum	6.11	6.11	6.49	6.49
Small intestine—				
Jejunum	6.31	6.50	6.62	6.48
Ileum	6.59	6.79	6.29	6.31
Caecum	6.52	6.36	6.94	7.01
Large intestine	6.50	6.61	6.88	6.99

We then carried out on albinos and piebalds a series of experiments similar to those previously done on guinea-pigs, in which normal animals on different normal diets were chloroformed at different periods after a meal, and the gastro-intestinal  $p_{\text{H}}$  determined. The diets fed varied from bread and milk to wheat, maize, oats and kitchen scraps (including greenstuffs), and contained adequate supplies of vitamins. Rats of different ages from 1 to 18 months were used. Typical results are shown in Fig. 2. The curve marked *K* is plotted from results which, by courtesy of Prof. W. C. McC. Lewis, were kindly obtained for us by Dr Millet, using the Kerridge [1925] glass electrode. It will be seen that they agree closely with those obtained by our own method.

In the adult rats little difference was found when feeding these different normal diets. This failure to produce any marked alteration in the intestinal  $p_{\text{H}}$  by varying the protein/fat/carbohydrate ratios of normal diets is in agreement with the findings of Abrahamson and Miller [1925], who fed adult albino rats different diets, some consisting largely of carbohydrate (boiled potato), some mainly protein (lean beef), and some mainly fat (beef fat), and obtained on the average only a variation of 0.1–0.2  $p_{\text{H}}$  in the stomach and small intestine. No results were given for the caecum and large intestine, and as their method was entirely colorimetric, the possibility of significant errors cannot be ignored. Their colorimetric results on a series of 36 animals were: stomach 2.6–4.1, average 3.9; duodenum and jejunum 5.2–6.4, average 5.8;

ileum 5.2-6.0, average 5.8. Our electrometric results on a series of 9 adult animals were: stomach 3.0-5.4, average 4.2; duodenum 4.0-5.7, average 5.2; jejunum 5.0-6.3, average 5.7; ileum 5.7-6.6, average 6.4; caecum 6.1-6.9, average 6.4; large intestine 6.9-6.9, average 6.4. As in the guinea-pig, the effect of varying the time between last meal and death was most marked in the stomach, and appeared to produce little difference elsewhere.

Half a dozen young normal rats (4-12 weeks old) on different normal diets were also examined. Typical results are indicated in Fig. 2 by broken lines, the age of each animal also being given. (In order to avoid complicating the figures, only a certain number of typical animals are included.) It will be seen that in young rats the small intestine apparently tends to be more alkaline than in adults (jejunum 6.5-7.2, average 6.8; ileum 6.3-7.6, average 7.0), but that the caecum and large intestine were always acid (6.5-6.9, average 6.7). From the recent work of Bergeim [1926] it appears that these last two sections of the gastro-intestinal tract are probably the most important in regard to calcium metabolism and the action of vitamin D.

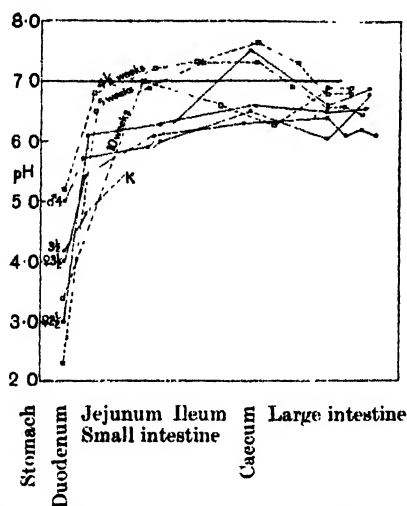


Fig. 2.  $p_H$  of gastro-intestinal tract of normal rats of different ages.

Young rats: --- □ ---. Adult rats: — ■ —.

The figure against each curve represents the number of hours between last meal and death.

Another question which arises is whether the  $p_H$  of the gastro-intestinal tract is in any way influenced by the nature of the intestinal bacterial flora. This problem has been carefully studied by Rettger and Cheplin [1921], working on both albino rats and human beings, and in both cases the conclusion was reached that the diet is the controlling factor. In regard to rickets, moreover, data obtained in experiments on dogs with various organisms isolated from the intestines of rachitic subjects would seem to render it unlikely that a bacteriological factor is predominant [Findlay, 1926]. It seemed to us that the fundamental fact to be investigated was the change in



faecal  $p_H$  which Zucker has found to be produced by the action of vitamin D, and, having found that in normal rats the  $p_H$  in the caecum and large intestine was always below 7.0, we decided next to examine the effect of withholding this vitamin.

An examination of the diets used by various workers to produce experimental rickets in rats showed that these can be classified in two main groups, according to their content of carbohydrate and fat. One, which has been employed in America by Sherman and Pappenheimer, Zucker and others is noticeable for its high carbohydrate content (nearly 70 %), and especially for its very low proportion of fat (less than 3 %) (see Table IV). The other, which has been principally used by British workers, contains rather less carbohydrate (50–60 %), and much more fat (15–17 %), and resembles much more closely in general composition the natural diet of infants. Since all the experiments so far recorded on the reaction of the faeces in rickets have been done on rats receiving the first "low fat" type of diet, we thought it would be of interest to ascertain if the change of faecal reaction to alkaline would also be obtained on withholding vitamin D from rats on a "high fat" diet.

Table IV. *Composition of diets used to test for vitamin D.*

	"Low fat" type	"High fat" type
Carbohydrate—		
Wheat starch (in flour)	69	—
Potato starch	—	56
Protein—		
Egg albumin and wheat protein	21.6	—
Caseinogen	—	23
Fat—		
Natural fat in wheat flour	1.7	—
Inactivated palm kernel oil	—	17
Salts—		
Calcium lactate	2.8	2.7
Ferric citrate	2.0	0.2
Sodium chloride	2.0	0.2
Other minerals	see note below	1.9
	99.1	99.0

Some workers use wheat starch in their "high fat" diets.

In certain "low fat" diets the protein is partly supplied as gelatin and maize, and the fat content may be a little higher (between 2 and 3 %).

Palm kernel oil is replaced by inactivated cotton-seed oil in certain "high fat" diets.

Variable amounts of phosphates of calcium, magnesium and potassium, and traces of silica and other minerals are present in wheat flour. The "low fat" diets are usually noticeable for containing a high percentage of sodium chloride (1 to 2 %).

The above figures are calculated on dry materials.

Vitamin A is usually supplied as spinach.

Vitamin B in the "low fat" diets is present in the cereal.

Vitamin C in the "high fat" diets is supplied as lemon juice.

In most of the previous studies on experimental rickets no account has been taken of the differentiation of the fat-soluble vitamins, and the animals have been deprived of vitamin A as well as of vitamin D. Chick and Roscoe [1926] appear to have been the first British workers to investigate the effect of depriving albino rats of vitamin D only, vitamin A being supplied daily in the form of fresh spring or early summer spinach leaves. The two diets

used were of the "high fat" type (15 and 17 %), and had calcium/phosphorus ratios of 0.4 : 1 and 0.7 : 1 respectively. No record was made of the intestinal reaction or the faecal  $p_H$ . Slight rickets was produced, the degree of calcification being nearer to normal than in animals also deprived of vitamin A. In these experiments, however, Chick and Roscoe were not always successful in avoiding xerophthalmia by the use of fresh spinach leaves, although the daily dose in some cases reached nearly 3 g. In view of these results, we decided to make certain modifications in Chick and Roscoe's technique when planning a similar investigation, in which the intestinal condition was also to be observed, and the relationship studied between faecal reaction and calcium and phosphorus retention. To ensure a satisfactory supply of vitamin A, whilst avoiding unduly bulky doses of spinach leaves, we prepared an acetone-ether extract of freshly dried early summer spinach, and have up to the present been able to secure freedom from xerophthalmia in all our experimental animals by supplying this extract in 25 mg. daily doses as the sole source of vitamin A. Further tests which are being carried out appear to indicate that 50 or 100 mg. doses of the extract do not contain significant amounts of vitamin D. In addition to using this spinach extract in place of the fresh leaves, we have employed a "high fat" type of diet whose calcium/phosphorus ratio more closely approaches the value 1.6 : 1 which is considered by McCollum [1923] to be approximately optimal for calcium absorption [cf. Willmott and Wokes, 1926, 1]. We hope to be able to publish later details of these studies.

The point of interest now is that when we had removed as far as possible all abnormal factors by supplying adequate amounts of vitamin A, and by using a diet with a normal fat content and a practically normal calcium/phosphorus ratio, we have still found that deprivation of vitamin D produces an alkaline faecal reaction, which changes back to acid on restoration of the vitamin. Daily tests over a number of weeks on the vitamin D-free diet have given an average faecal  $p_H$  of 7.3, with fluctuations from 6.5 to 7.8.

In order to satisfy ourselves that the basal diet itself is not responsible for producing the alkaline condition, we have fed it to normal rats receiving adequate supplies of vitamins A and D, vitamin B being supplied in the form of a yeast extract, and have examined the intestinal reaction after different periods. The results, which are shown in Fig. 3, would seem to prove that the diet tends to produce in the gastro-intestinal tract a condition which is more acid than normal, probably on account of the type of salt mixture employed.

In regard to the faecal  $p_H$  in normal rats, it is of interest to note that Rettger and Cheplin [1921] when examining 146 samples from albinos on different diets, found them always acid.

We have also paid attention to the "low fat" type of diet. Thirteen different young albinos and piebalds which were fed this diet (see Table IV) developed a definitely alkaline faecal reaction ( $p_H$  7.3-8.0) in 10 to 14 days. Three of these animals, when their faeces had remained at about  $p_H$  8.0 for at least a week, were chloroformed and examined. The results, which are

shown as continuous lines in Fig. 4, indicate a definite tendency towards greater alkalinity throughout the tract. Even the stomach, after digestive activity had subsided, was only faintly acid ( $p_H$  6.1–6.5). In one animal the most alkaline point reached was only 7.2, but in the other two a reading of 7.8 was recorded. At the lower end of the large intestine these two gave slightly higher readings (7.3 and 7.4) than the less alkaline animal (7.2), so that, judging by these three cases, the  $p_H$  of the faeces in rats on Zucker's diet would appear to give a fairly reliable indication of the conditions higher up the tract, in those parts where calcium absorption is probably most important. Abrahamson and Miller [1925], using colorimetric methods, also found that deprivation of vitamin D raised the  $p_H$  from 6.0 to 7.0 in the jejunum, and from 6.0 to 7.1 in the ileum. Unfortunately the colorimetric results were not checked by electrometric methods, and no readings were taken in the caecum or large intestine. On administration of cod-liver oil, however, the original acid condition was restored.

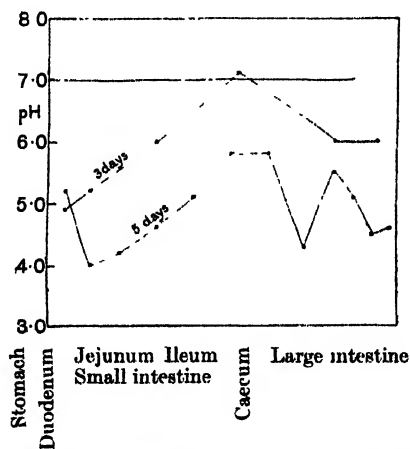


Fig. 3. Effect of "high fat" type of rachitogenic diet on  $p_H$  of gastro-intestinal tract of normal rats receiving vitamin D.

Autopsies on our three rats did not reveal any marked signs of rickets, which was perhaps not surprising in view of the short period they had been on the rachitogenic diet. Microscopic and chemical examinations of bones from animals which have been kept on the diet for a longer period are now being made, and would appear to indicate the incipient stages of the disease. The condition of the animals is, in general, very similar to that which McCollum obtains in the preparatory period for his "line" test [McCollum, Simmonds, Shipley and Park, 1922].

On administering to the animals whose faecal  $p_H$  had remained at about 8.0 for 14 days vitamin D as cod-liver oil or as irradiated cholesterol, we found that the faecal  $p_H$  dropped gradually to about 6.0. The rate at which it drops seems to be influenced by the amount of vitamin D supplied, but if this is too large the faeces become very loose. In the case of four animals which had

been on exactly the same treatment, for the same period, administration of the same amount (about 0.15 g.) of a Norwegian oil brought the  $p_H$  down at approximately the same rate, the greatest variation in  $p_H$  of faeces (which were collected separately from each animal) on any day being 0.5. Taking the faeces of any one animal on any given day, the greatest variation we have found in a number of samples is 0.2, and often it is much less. For this reason we suggest that an accuracy of 0.1 in the  $p_H$  determination is sufficient for the Zucker method.

We have also tested the effect of adding to the Zucker diet a well buffered natural food substance. For this purpose we used the freshly expressed juice of ripe Californian navel oranges (Sunkist brand). This had an average  $p_H$  of 4.2, and was so well buffered that 12 to 15 cc. of *N* NaOH were required to bring 100 cc. to  $p_H$  7. Four albino rats were put on a Zucker diet, and vitamin A was supplied as a daily ration of 2-3 g. fresh spinach leaves. When the faeces had been alkaline for over a week, each animal was then given a daily dose of 5 cc. of fresh orange juice. Since one of us had found this amount of juice to contain adequate amounts of vitamin A (unpublished data) the spinach was then discontinued.

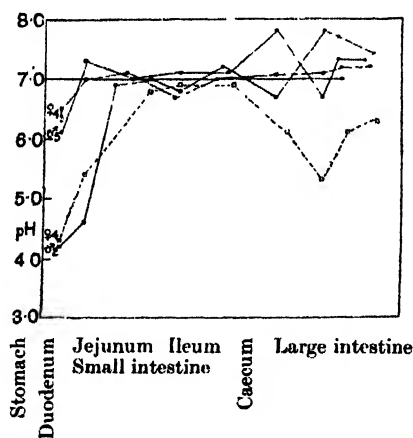


Fig. 4. Effect of vitamin D on  $p_H$  of gastro-intestinal tract of young albino rats on Zucker's "low fat" diet.

Rats on vitamin D-free diet: ——— • ———. Rats receiving D: - - - □ - - -. The figure against each curve represents number of hours between last meal and death.

Daily readings taken for over a fortnight showed that the faecal  $p_H$  still remained alkaline (7.5-8.0). Vitamin D was then administered in two different ways, as irradiated cholesterol and as cod-liver oil, and in each case the faeces gradually became acid. One of the animals, when its faeces had been acid for 5 days, was then chloroformed and examined. The results, which are shown as broken lines in Fig. 4, indicate that administration of vitamin D had restored the acid condition throughout the gastro-intestinal tract.

*Experiments on other rodents.*

Mice are not frequently used in feeding experiments on vitamins, although they were employed in the pioneer investigations of Stepp [1909]. Some white mice kindly supplied by Sir F. G. Hopkins were examined. The results would seem to show that the reaction in the gastro-intestinal tract of the white mouse on a bread and milk diet is similar to that of the rat, but somewhat more acid, the highest  $p_H$  recorded being under 6.0. The amounts of material available, however, were so small that the results are not considered sufficiently accurate for publication of details.

Rabbits have recently been used by Mellanby and Killick [1926] as experimental animals for the production of rickets, being fed on a basal diet of bran and oats, with turnip to supply vitamin C. We carried out an experiment on a young rabbit which had been fed on a diet of bran and oats, with cabbage to supply vitamins A and C. The results, which are summarised in Fig. 5, resemble those previously obtained on the guinea-pig, another herbivorous animal.

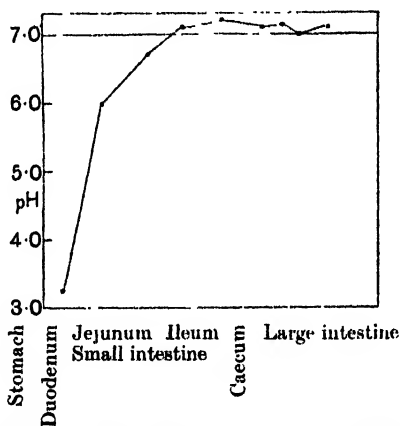


Fig. 5.  $p_H$  of the gastro-intestinal tract of a normal adult rabbit.

## DISCUSSION.

Although this investigation is in various respects incomplete, we think it desirable to mention certain points which may help to elucidate our experimental results, and perhaps indicate new lines of attack. Dealing first with the rat, on which the bulk of our work has been done, we have shown that deprivation of vitamin D produces in the gastro-intestinal tract a greater tendency towards alkalinity, and restoration of the vitamin brings the reaction back to acid. In the caecum and large intestine the difference between rats receiving vitamin D, and those deprived of it, is even more marked, a change in  $p_H$  of such a small range as 6.6 to 7.4 appearing to be significant. Assuming for the moment that the incidence of rickets is influenced by the degree of calcium absorption in the intestines, it is obvious that this in turn will be subject to various factors which affect the solubility of calcium. Under normal

conditions, the gastric hydrochloric acid probably is sufficient to render into soluble form practically the whole of the calcium in the food, as has been shown by Telfer [1924] in experiments both *in vitro* and *in vivo*, using dogs as subjects. As the food passes down the intestine, the gastric acidity tends to be more or less neutralised by the bile and other alkaline secretions, and calcium may be precipitated as carbonate, as soaps or as phosphates [cf. Willimott and Wokes, 1926, 2]. Blauberg [1900] has published analyses of the faeces of rachitic children, indicating that in rickets an abnormal amount of calcium is excreted in combination with phosphorus. Robison [1923] has demonstrated the presence in the wall of the small intestine in rats, guinea-pigs and rabbits of the phosphoric esterase which he suggests performs an essential part in calcification in the bones by liberating phosphate ions, and thus precipitating calcium phosphate. Robison and Soames [1924], using a preparation of this enzyme to hydrolyse different phosphoric esters, have shown that it has an optimum  $p_H$  range of 8.4-9.4, and rapidly loses its activity as the  $p_H$  falls from 7.5 to 6.8. Provided that this enzyme is present in the intestine, the suggestion arises that the change in faecal and intestinal reaction from alkaline ( $p_H$  7.2-7.8) to acid ( $p_H$  6.5-6.8) which we have shown to occur in rachitic rats after administration of an antirachitic substance, may, by retarding the action of this phosphoric esterase, diminish the precipitation of calcium phosphate and thus facilitate calcium absorption from the intestines. If such is the case, then a metabolism experiment should show that the calcium retention is greatest when the faeces are acid, and least when they are alkaline, provided that the reaction of the faeces is a reliable indication of the  $p_H$  of the parts of the intestines most particularly concerned with calcium absorption and retention. In regard to the latter point, the recent results of Bergeim [1926] are significant. By means of experiments on rats, he found that on a rachitogenic diet the absorption of calcium is most seriously affected in the caecum and large intestine, where it might even become negative.

When vitamin D was administered in the form of cod-liver oil, at the level of 10 drops a day, the following increases were observed in the average percentage absorption of calcium: in the jejunum, an increase from 63 to 73, in the ileum 35 to 57, in the caecum nil to 50, in the large intestine minus 5 to 80. It is interesting to compare these results, which were obtained on a "low fat" type of diet, with the intestinal  $p_H$  we found in the three rats on the Zucker "low fat" rachitogenic diet. In all three cases the most alkaline point reached was not until the lower end of the small intestine, and in two cases there was only significant alkalinity ( $p_H$  7.2-7.8) in the caecum and large intestine.

These results having led us to suppose that the faecal  $p_H$  can be considered a reliable indication of the intestinal reaction, we have set in train metabolism experiments in which the calcium and phosphorus retentions are being measured in rats fed different diets deficient in vitamin D, and compared with daily variations in the faecal  $p_H$ . We hope to be able to publish later details of our findings.

In regard to guinea-pigs, the fact that we have shown that the greater portion of the small intestine is normally definitely alkaline ( $p_H$  7.2–7.8), while the caecum and large intestine are acid ( $p_H$  6.8–5.2), would seem to suggest that in this animal also a significant amount of calcium absorption takes place in the caecum and large intestine.

In regard to human beings, Flamini [1926] has recently reported results of experiments in which he found that the administration of vitamin D concentrate (ostelin) to rachitic babies was followed by a change in the reaction of the faeces from alkaline to acid, the "titratable acidity" for 100 cc. of faeces altering from 100–150 cc.  $N/10$   $H_2SO_4$  to 100–300 cc.  $N/10$   $NaOH$ . Observations are now being made with the object of ascertaining if the reaction of the faeces in children can be correlated with the rachitic condition as it apparently can be in rats.

#### SUMMARY.

Colorimetric and electrometric methods for determining the  $p_H$  of the gastro-intestinal tract were examined, and an estimate made of the errors due to protein and salts, dilution and loss of  $CO_2$ . A satisfactory technique was evolved using a capillary electrode vessel.

In guinea-pigs the following average  $p_H$  results were obtained: stomach 2.8–4.3, average 3.8; duodenum 4.8–5.3, average 5.1; jejunum 5.1–7.1; ileum 7.0–7.9, average 7.4; caecum and large intestine 5.2–6.9, average about 6.4.

In normal adult rats (albinos and piebalds) the average figures were: stomach 4.0, duodenum 5.5, jejunum 6.0, ileum 6.8, caecum and large intestine 6.5.

In rats 1–3 months old the average figures were: jejunum 6.8, ileum 7.0, caecum and large intestine 6.7.

A "high fat" type of rachitogenic diet when fed to rats gave greater acidity to the faeces and throughout the intestinal tract if vitamin D was supplied, but in the absence of vitamin D the faecal reaction became alkaline.

Rats fed on Zucker's "low fat" rachitogenic diet developed alkalinity throughout the intestinal tract, but especially in the caecum and large intestine (average  $p_H$  7.3), which from Bergeim's work would appear to be the most significant areas in calcium absorption and retention. Administration of vitamin D as cod-liver oil or as irradiated cholesterol restored the acidity.

On account of increased buffering in the caecum and large intestine, the  $p_H$  of the faeces, which has been suggested as a criterion for the estimation of vitamin D, would appear to be a reliable indication of the reaction in these sections of the gastro-intestinal tract, at least in the case of the rat.

Zucker's method for testing for vitamin D has been applied to a buffered natural food substance (orange juice).

The mouse and rabbit were also examined.

A possible relationship is discussed between the intestinal  $p_H$  and the occurrence of a phosphoric esterase in the aetiology of rickets.

We are indebted to Sir F. G. Hopkins for his advice and interest in the work, to Prof. J. M. Beattie for laboratory facilities, and also for kindly diagnosing the condition of the animals, and to the scientific staff of Messrs Joseph Nathan and Co. for affording us free access to their experimental results.

A grant towards the expenses of the investigation was received from the Pharmaceutical Society of Great Britain.

Part of the work was done under the tenure of the Virol Research Scholarship in Clinical Pathology by one of us (T. R.).

#### REFERENCES.

- Abrahamson and Miller (1925). *Proc. Soc. Exp. Biol. Med.* **22**, 438.  
Bergeim (1926). *J. Biol. Chem.* **70**, 20.  
Biilmann (1921). *Ann. Chem.* **15**, 109.  
Biilmann and Lund (1921). *Ann. Chem.* **16**, 321.  
Blauberg (1900). *Z. Biol.* **40**, 1.  
Chick and Roscoe (1926). *Biochem. J.* **20**, 137.  
Clark (1920). The determination of hydrogen ions.  
Corran and Lewis (1924). *Biochem. J.* **18**, 1358.  
Cullen and Biilmann (1925). *J. Biol. Chem.* **64**, 727.  
Donaldson (1924). The rat (Philadelphia), p. 122.  
Findlay (1926). *Private communication*.  
Flamini (1926). *La Pediatra*, **34**, 625.  
Henderson and Palmer (1912). *J. Biol. Chem.* **13**, 393.  
Jephcott and Bacharach (1926). *Biochem. J.* **20**, 1351.  
Kahn and Stokes (1926). *J. Biol. Chem.* **69**, 75.  
Kerridge (1925). *Biochem. J.* **19**, 611.  
Læpper and Martin (1926). *Biochem. J.* **20**, 45.  
Læpper and Zilva (1925). *Biochem. J.* **19**, 581.  
Long and Fenger (1917). *J. Amer. Chem. Soc.* **i**, **39**, 1278.  
McClendon (1915). *Amer. J. Physiol.* **38**, 180.  
McCollum (1923). The Newer Knowledge of Nutrition. (New York.)  
McCollum, Simmonds, Shipley and Park (1922). *J. Biol. Chem.* **51**, 41.  
Mellanby and Killick (1926). *Biochem. J.* **20**, 902.  
Rettger and Cheplin (1921). A treatise on the transformation of the intestinal flora. (Yale University Press.)  
Robinson (1925). *J. Biol. Chem.* **66**, 811.  
Robison (1923). *Biochem. J.* **17**, 286.  
Robison and Soames (1924). *Biochem. J.* **18**, 740.  
Stepp (1909). *Biochem. Z.* **22**, 452.  
Telfer (1924). *Quart. J. Med.* **17**, 245.  
Willmott and Wokes (1926, 1). *Pharm. J.* Oct. 23, 495.  
—— — (1926, 2). *Pharm. J.* Oct. 30, 521.  
Zucker and Matzner (1924). *Proc. Soc. Exp. Biol. Med.* **21**, 186.



# LXXXIV. STUDIES IN THE SULPHUR METABOLISM OF THE DOG.

## V. TOXIC ACTION OF MERCAPTURIC ACIDS.

By ERNEST HAROLD CALLOW AND THOMAS SHIRLEY HELE.

*From the Biochemical Laboratory, Cambridge.*

*(Received May 2nd, 1927.)*

So far as has been ascertained there are only two records in the literature of the administration of mercapturic acids to dogs. Baumann and Preusse [1881] administered by mouth 8 g. of the ammonium salt of *p*-bromophenylmercapturic acid. No ill effects were observed; no glycuronic acid was excreted and only traces of mercapturic acid. No sulphur analyses apparently were done. Marriott and Wolf [1907] gave 1 g. of the bromo-acid by mouth. No ill effects were recorded but in the record the sulphur analyses of the urine do not show clearly whether any mercapturic acid was excreted. Possibly absorption was incomplete in both cases.

In the year 1922 during the course of experiments recorded in Part III of this series [Callow and Hele, 1926] it was found quite unexpectedly that an oral dose of 1 g. of *p*-bromophenylmercapturic acid produced a transient haemoglobinuria in the dog "Vixen." This result has been confirmed in several other dogs.

### EXPERIMENTAL.

The methods employed in this research were identical with those already described [Callow and Hele, 1926]. Five dogs were used. "Vixen" and "Patricia" received the diets mentioned previously. "Diana" was not placed on a standard diet. "Phoena" and "Pansy" were given the following diets:

"Phoena" (6.97 kg.):

		T.N. g.	T.S. g.	Calories K
Lean meat	80 g.	2.73	0.181	106
Margarin	40 g.	—	—	312
Arrowroot biscuit	20 g.	0.22	0.025	121
Milk	160 cc.	0.83	0.074	115
Total		3.78	0.280	654

"Pansy" (8.62 kg.):

		T.N. g.	T.S. g.	Calories K
Lean meat	80 g.	2.73	0.181	106
Margarin	20 g.	—	—	156
Sucrose	50 g.	—	—	205
Milk	160 cc.	0.83	0.074	115
Total		3.56	0.255	582

*Exp. 1. "Vixen."* At 11 a.m. 1 g. *p*-bromophenylmercapturic acid was administered by mouth. At 5 p.m. on the same day the urine voided was almost black in colour and contained much haemoglobin. The microscope showed the sediment to consist of red-cell debris. There was evidence neither of haematuria nor of a primary nephritis. At 5 p.m. on the following day the urine was free from haemoglobin. Traces of albumin however persisted for another two days.

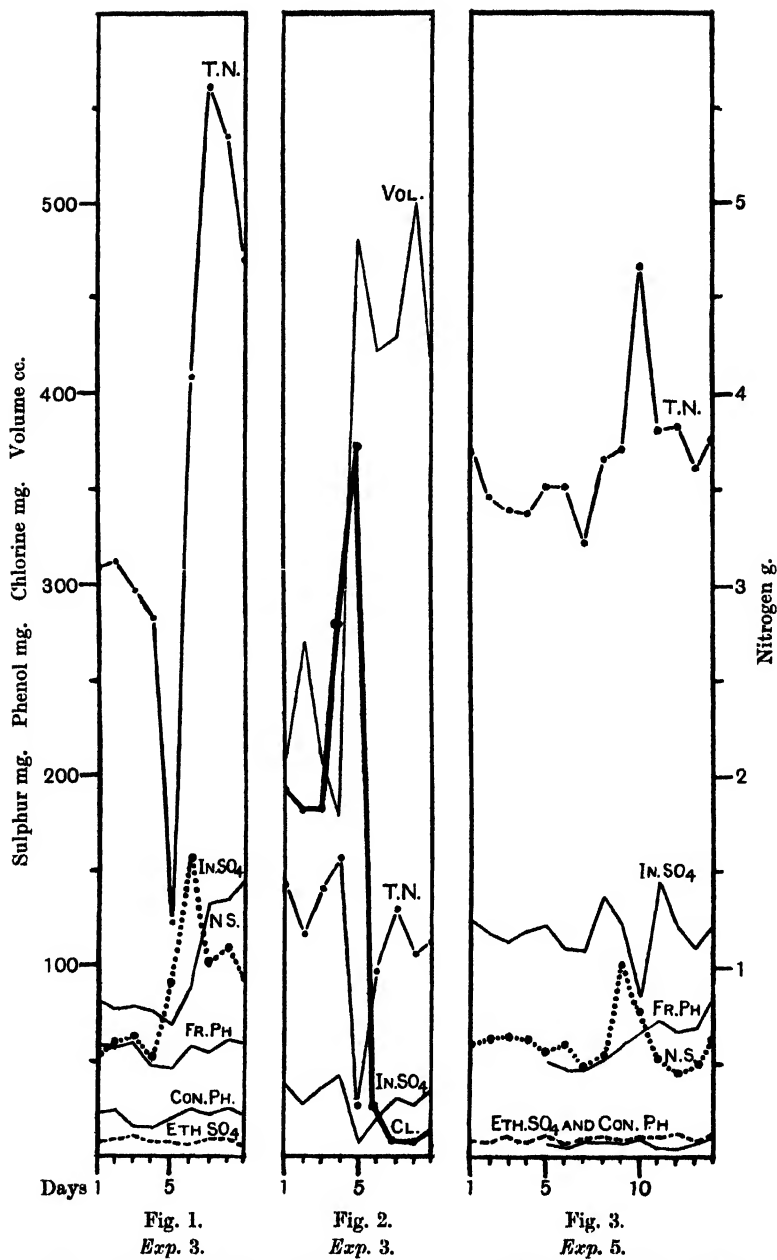
*Exp. 2. "Patricia."* 0.5 g. *p*-bromophenylmercapturic acid was administered by mouth. No ill effects were noted. The dose was too small to make any appreciable alteration in the output of neutral sulphur.

*Exp. 3. "Phoena."* At 11 a.m. 2.5 g. *p*-chlorophenylmercapturic acid were administered by mouth. At 9 a.m. on the following day 15 cc. of deep red urine were removed from the bladder, the previous urine being clear. The red urine contained much free haemoglobin, much cell debris and a few blood cells. At 12.45 p.m. there was in the urine only a trace of haemoglobin with a few blood cells. Until 9 p.m. there persisted a trace of albumin but no further trace of haemoglobin. The dog vomited the evening meal on the first day and afterwards refused all food except the meat and milk. In consequence the outputs of nitrogen and of neutral sulphur, which rose considerably, had no experimental value. There was however no increase in the outputs of ethereal sulphate, free or conjugated phenols, indicating that there had been no conversion of mercapturic acid to ethereal sulphate. The "organic" chlorine excreted in the first 24 hours after the dose corresponded to only 6 % of the dose, but on the following day during the recovery period the organic chlorine indicated an excretion of 30 % of the dose. Although chlorides were freely excreted during the haemoglobin period yet there was a marked retention of nitrogen. Afterwards the chloride excretion fell and the nitrogen rose. The excretion of sulphate was similar to that of the nitrogen. Fig. 1 shows graphically the excretives in the urine during the experimental period. Fig. 2 shows the concentration of chloride (expressed as chlorine), total nitrogen and sulphate in the urine and also the variations in its volume, associated with variations in the water intake.

*Exp. 4. "Diana."* 0.5 g. *p*-chlorophenylmercapturic acid was given subcutaneously as the ammonium salt. No ill effects were noted.

A week later 1 g. *p*-bromophenylmercapturic acid was given subcutaneously as the ammonium salt at 10 a.m. At 10 p.m. there was a trace of haemoglobin in the urine. The urine voided during the night was very dark coloured and the haemoglobin persisted until 2.30 p.m. on the following day. After 4.30 p.m. the urine contained no albumin. During the haemoglobin period the sediment contained much cell debris with a very occasional blood cell or epithelial cell. There were no casts until the recovery period and then only a few epithelial and blood casts.

*Exp. 5. "Pansy."* This experiment is shown in Fig. 3. At 10 a.m. 1.23 g. *p*-bromophenylmercapturic acid was administered by the mouth. The urine



Figs. 1 and 2. "Phoena." Day No. 5. 2.5 g. *p*-chlorophenylmercapturic acid.  
 Fig. 3. "Pansy." „ No. 9. 1.23 g. *p*-bromophenylmercapturic acid.

remained clear until 5 p.m., when the dog was placed in the metabolism cage. The urine passed during the night was mostly clear, though there was a little blood at the bottom of the collecting vessel. At 9 a.m. on the following day 17 cc. of bloody urine were removed from the bladder. This urine contained much cell debris, only a few red cells and no casts. For the quantitative work this urine was discarded. For the next 24 hours the urine was cloudy, containing traces of blood and some albumin. On the third day the urine was clear and free from albumin. The diet was maintained throughout, so that variations in the quantities of the urinary products could not be ascribed to errors of diet. During the "albumin" period estimations in duplicate were carried out on the untreated urine, on the urine rendered protein-free by trichloroacetic acid and on the urine rendered protein-free by "dialysed iron." The agreement between these was very close, showing that the quantity of albumin present was too small to affect the neutral sulphur figure appreciably. The rise in neutral sulphur corresponded to an excretion of 32 % of the dose in the first 24 hours and 16 % in the second. There was no retention of nitrogen as in Exp. 3, showing that the permeability of the kidney was hardly affected. There was no alteration in the outputs of ethereal sulphate or conjugated phenols, indicating that there had been no conversion of mercapturic acid to ethereal sulphate. No explanation is offered for the rise in the output of nitrogen on the tenth day and of inorganic sulphate on the eleventh day. The rise in free phenols was slow and continuous and bore no relation to the dose-day.

#### DISCUSSION.

From the above experiments it is clear that the administration of *p*-bromo- or chloro-phenylmercapturic acid may produce a transient haemoglobinuria in the dog. There is a minimum dose necessary to bring about this effect. If the haemoglobinuria is considerable and the kidney tubules are largely blocked with cell debris, there is a retention of nitrogen and the excretion of the mercapturic acid is delayed. These experiments would suggest that this effect of mercapturic acid might be used as a method of producing experimentally a variable degree of "blocked kidney," of the kind observed in blackwater fever. The cause of the haemolysis which precedes the excretion of the haemoglobin is not clear. The effect is observed, whether the mercapturic acid is administered orally or hypodermically. Preliminary experiments on the direct action of mercapturic acids on the blood cells of the dog *in vitro* suggest that mercapturic acids have no direct haemolysing action. The action of mercapturic acids is remarkable, when the comparatively non-toxic character of bromo- and chloro-benzene is considered. These dogs are capable of dealing with 5 g. of bromobenzene in a single dose without haemolysis being produced, that is ten times the dose of bromobenzene equivalent to the toxic dose of the corresponding mercapturic acid. Of course the concentration of mercapturic acid in the effective organ may never be so high

at any given moment after bromobenzene as after the administration of the mercapturic acid. Until, however, something is known about the mechanism of the production of the haemolysis, it would be wrong to regard the formation of mercapturic acid in the dog as a protective synthesis.

It must be noted that in these experiments there is no indication of any conversion of mercapturic acid to ethereal sulphate. Any such conversion would be seen in the outputs of ethereal sulphate and of conjugated phenols.

#### SUMMARY.

1. *p*-Bromo- and *p*-chloro-phenylmercapturic acids, whether given by the mouth or subcutaneously, cause a transient haemoglobinuria in the dog. There is a minimum dose required to produce this effect.

2. There is no evidence of any conversion of mercapturic acids to ethereal sulphates in the dog.

The authors desire to express their thanks to the Government Grant Committee of the Royal Society for grants in aid of this research, and one of them (E. H. C.) to the Department of Scientific and Industrial Research for a personal grant.

#### REFERENCES.

- Baumann and Preusse (1881). *Z. physiol. Chem.* 5, 339.  
Callow and Hele (1926). *Biochem. J.* 20, 598.  
Marriott and Wolf (1907). *Biochem. Z.* 7, 244

## LXXXV. STUDIES IN THE SULPHUR METABOLISM OF THE DOG.

### VI. THE METABOLISM OF THE PIG AND DOG COMPARED<sup>1</sup>.

BY HERBERT ISAAC COOMBS AND THOMAS SHIRLEY HELE.

*From the Biochemical Laboratory, Cambridge.*

*(Received May 2nd, 1927.)*

IN a former communication one of us [Hele, 1924, 1] was able to show that ethereal sulphates are formed by direct union of phenolic compounds with preformed sulphate. This work is directly opposed to the views held by Shiple, Muldoon and Sherwin [1924], who have published an account of work, which they interpret as evidence of the formation of ethereal sulphates from some product of endogenous sulphur metabolism, something of the type of a mercapturic acid being the intermediary. It seemed to us that perhaps this difference of opinion was caused by a difference in the species of animal employed, because, whereas dogs were used in this laboratory, a pig was used in the experiments of Shiple, Muldoon and Sherwin. We therefore decided to study the metabolism of the pig and to repeat as far as possible the experiments, which have been described in the former papers of this series and in which the dog was used as the experimental animal.

Early in our work it became obvious that there are great differences in the metabolism of the pig and of the dog. We found that the pig, unlike the dog, does not readily detoxicate phenolic substances by combination with compounds containing sulphur. The increase in the reducing power of the urine would suggest that the detoxicating agent is glycuronic acid. This then we believe to be the essential difference. Whereas in the dog ethereal sulphates and mercapturic acids are formed readily and glycuronic acids less readily, in the pig glycuronic acids are formed readily, ethereal sulphates less readily and mercapturic acids probably not at all. When our work was extended to the metabolism of the mercapturic acids, we also found a difference between the two species. No evidence could be obtained of a conversion of mercapturic acids to ethereal sulphates. In this respect the pig resembles the dog. On the other hand, with the doses used, mercapturic acids produced no sign of the haemoglobinuria which is so remarkable in the dog.

<sup>1</sup> The previous papers of this series are numbered 1—5 in the list of references.

## EXPERIMENTAL.

Two experiments were carried out on different animals, pure bred female pigs, recently weaned, of the "Large White" breed. Young pigs had to be used, as there were no facilities in the laboratory for using adult pigs. An attempt was made to catheterise the pigs daily, a procedure so valuable in metabolism experiments on the dog. In the young pig this operation is difficult and was soon abandoned in favour of changing the pig from cage to cage every few hours, as each cage became fouled. The 24-hours' urine was made up each morning at 9 a.m. The volume was always large (1500 to 2000 cc. as compared with the 200 to 300 cc. of the dog). We found the pig's habits to be very regular and as any error in collection would be of urine passed many hours after food and therefore of low sulphur content, we thought failure to catheterise was not a serious matter. The analytical figures obtained justified this practice.

The diets used were as follows:

*Exp. 1 (Fig. 1):*

"Phyllis," born May 8th. Experiment July 16th to August 23rd, 1926. Weight at commencement of experiment, 14.5 kg.; at end, 18.5 kg.

9 a.m.	Sharps	175 g.	Water 1000 cc.
12 noon	Grey peas	200 g.	Fed dry
5 p.m.	Sharps	175 g.	Water 1000 cc.

The nitrogen content of the diet was 15.4 g.

*Exp. 2 (Fig. 2):*

"Rosamund," born July 23. Experiment October 29th to November 20th, 1926. Weight at commencement of experiment, 17.7 kg.; at end, 18.6 kg.

9 a.m.	Sharps	250 g.	Water 1500 cc., cabbage 100 g.
12 noon	Grey peas	200 g.	Fed dry
5 p.m.	Sharps	250 g.	Water 1500 cc., cabbage 100 g.

The nitrogen content of the diet was 19.6 g.

The "Sharps" were soaked for 24 hours in half the ration of water and the remainder of the water was added at the time of feeding. The sodium sulphate and guaiacol carbonate used in Exp. 1 were mixed with the morning and evening food. The remaining substances were administered in gelatin capsules. At first we had considerable difficulty in getting the pig to swallow the capsules but later, using the following technique, we were able to give the various doses easily and with certainty.

The pig is held with the fore feet off the ground between the knees of an assistant, who is in a standing position. A piece of thin rope is tied round the upper jaw behind the snout and the snout is drawn upwards and backwards. Under these circumstances the pig keeps the mouth open. The operator then draws the tongue forward preferably with forceps and throws or blows through a tube the capsule to the back of the tongue. The tension on the rope is immediately released and the pig swallows the capsule. The assistant should place a stick between the teeth on one side of the pig's mouth to prevent it accidentally biting the operator. If available a second assistant can be employed in holding the pig's back legs off the ground.

All the chemicals that were administered were of the highest degree of purity. The guaiacol carbonate, phenol, bromobenzene and iodobenzene were obtained from Kahlbaum, the *p*-iodophenol was made from *p*-aminophenol by the method of Brazier and McCombie [1912] and the mercapturic acids were prepared from the urine of dogs by the usual method.

The analytical methods have all been previously described [Hele, 1924, 2] except those for the determination of iodine. For this the method described by Kendall [1920] proved very satisfactory. We preferred however to incinerate with sodium peroxide instead of with sodium hydroxide.

#### *The formation of ethereal sulphate.*

We tried to repeat the experiments described in the first paper of this series [Hele, 1924, 1], using the pig in place of the dog. Larger doses were used of sodium sulphate (4 g. daily) and of guaiacol carbonate (10 g. on each of two days), as the normal output of inorganic sulphate was larger in the pig (Exp. 1, Fig. 1, days 5 to 13). About 80 % of the sulphate was absorbed and excreted. The guaiacol carbonate, however, gave rise to only a minute amount of ethereal sulphate, the excess over the normal daily output of ethereal sulphate-sulphur on the two days being respectively 14 and 27 mg. S and on the day following 34 mg. S. Estimation of the phenolic substances in the urine by the colorimetric method [Folin and Denis, 1915, 1916] suggested that there were being excreted each day about 2 g. of the guaiacol, equivalent to about 500 mg. sulphur. As guaiacol is rather insoluble, we tried phenol (3 g., Fig. 1, day 11). The excess ethereal sulphate-sulphur on the phenol day was only 74 mg. In the dog this dose of phenol would have yielded an excess of at least 400 mg. The colorimetric method indicated an excretion of about 2 g. of the phenol in the urine, equivalent to 680 mg. S.

It therefore became obvious that the experiment, which proved so successful on the dog, could not be repeated on the pig, because so small an amount of ethereal sulphate was formed in the latter case. To prove the point at issue it is not sufficient merely to produce a rise in ethereal sulphate larger than the rise following the administration of the phenol alone, but it is necessary to produce such a rise that it is immediately clear that some of the administered sulphate has been utilised. It would have required at least 18 g. phenol, too large a dose for safety, to convert all the inorganic sulphate of food and tissue origin and some of the administered sulphate to ethereal sulphate. Quite apart from any direct action on sulphur metabolism such a dose, even if given successfully, would probably have caused an increase in tissue breakdown. The rise in the output of sulphate from this source would then have obscured the issue.

Subsequently we gave 10 g. guaiacol carbonate alone to the same pig (Fig. 1, day 25) and again obtained only a minute rise in the ethereal sulphate, a rise too small for any comparison with the previous guaiacol experiment. To the second pig (Fig. 2, day 18) we administered 4.4 g. *p*-iodophenol. We



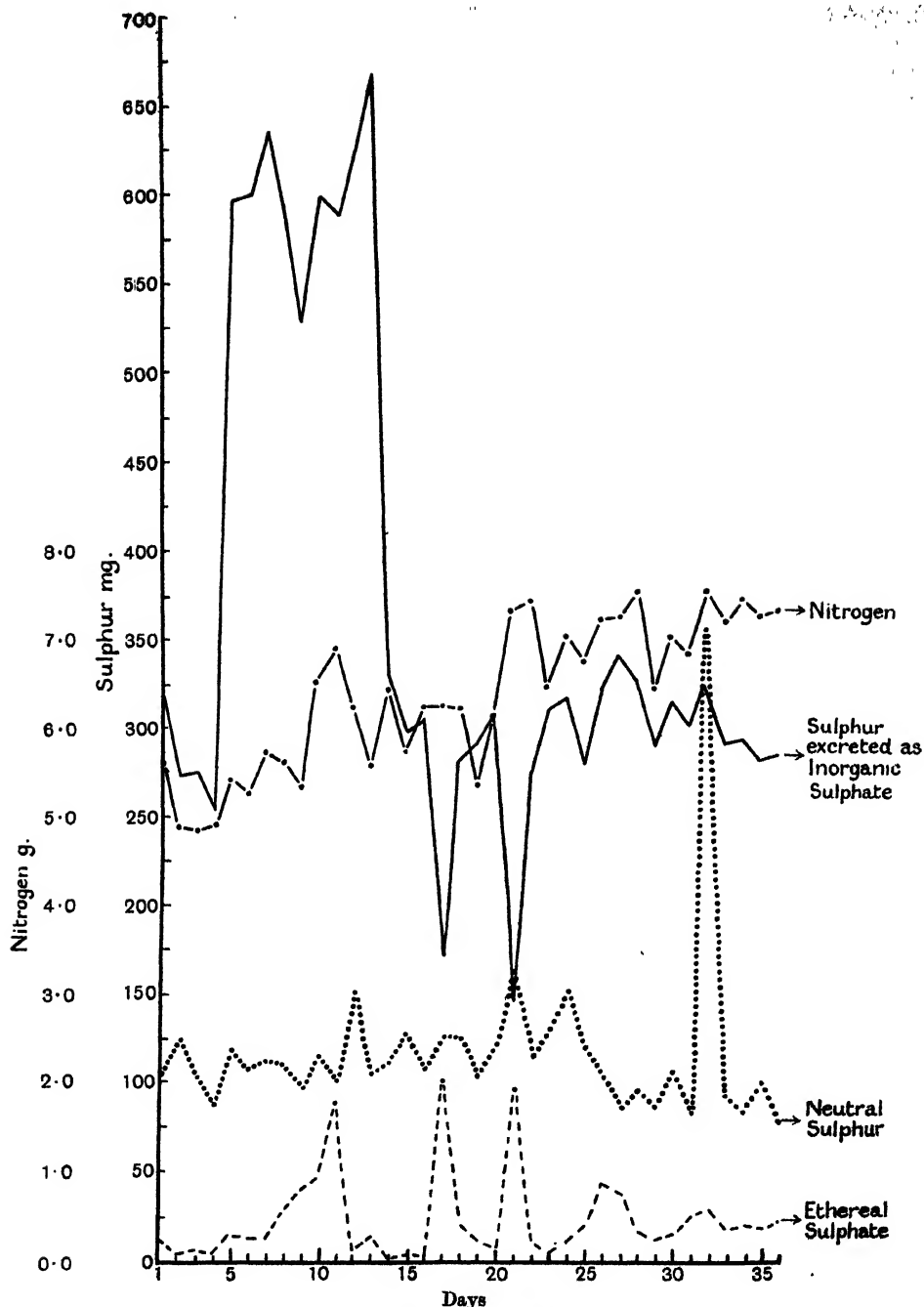


Fig. 1. "Phyllis."

Days No. 5 to 13. 4 g.  $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$  daily.

" No. 8 and 9. 10 g. guaiacol carbonate daily.

" No. 11. 3 g. phenol.

" No. 17. 4.08 g. ( $2 \times \text{m.w.}/100 \text{ g.}$ ) iodobenzene.

" No. 21. 3.14 g. ( $2 \times \text{m.w.}/100 \text{ g.}$ ) bromobenzene.

" No. 25. 10 g. guaiacol carbonate.

" No. 32. 3.65 g. ( $1 \times \text{m.w.}/100 \text{ g.}$ ) *p*-iodophenylmercapturic acid.

This figure may be compared directly with the figures in Papers III, IV and V of this series. The figure in Paper I is, however, drawn to a different scale.

used the iodine compound as the output of iodine in the urine afforded an indication of absorption and excretion. From the iodine figures it was clear that 76 % of the dose was excreted on the first day and 8 % on the second day. The ethereal sulphate analyses indicated an excretion of only 10 % of the dose in this form. The dogs "Vixen" and "Sophia" excreted 59 % and 53 % respectively of the corresponding chloro-compound in the form of ethereal sulphate [Coombs and Hele, 1926]. These results are summarised in Table I.

Table I. *The formation of ethereal sulphate.*

Percentage of dose of various compounds excreted as:

	Ethereal sulphate		Inorganic sulphate	
	Pig %	Dog %	Pig %	Dog %
Sodium sulphate alone	Nil	Nil	81	89
Guaiacol carbonate alone	2.5	12	Nil	Nil
Guaiacol carbonate and sodium sulphate together:				
Guaiacol carbonate	2.4	30	Nil	Nil
Sodium sulphate	Nil	66	69	22
Phenol alone	—	38	—	Nil
		57		
<i>p</i> -Chlorophenol alone	—	53	—	Nil
		59		
<i>p</i> -Iodophenol alone	10*	—	Nil	—
Phenol and sodium sulphate together:				
Phenol	8	—	Nil	—
Sodium sulphate	Nil	—	75	—

The numbers for the dog are taken from Papers I and IV of this series.

\* The iodine excreted in the urine indicated that 84 % of the *p*-iodophenol was excreted.

It is clear from these experiments that ethereal sulphates are less readily formed in the pig than in the dog. We have no proof of the fate of the large balance of the absorbed phenols. The urine of course contained glycuronic acid and the increase in the reducing power of the urine suggested that the balance of the phenols was excreted combined with this substance.

In no instance was any rise in neutral sulphur observed in the urine after the administration of phenols; in this respect the pig resembles the dog.

#### *The effect of the monohalogenbenzenes on metabolism.*

We administered 4.08 g. iodobenzene to the first pig (Fig. 1, day 17) but did not obtain any rise in the neutral sulphur of the urine. On the dose-day there was a rise of 98 mg. S in the ethereal sulphate fraction and 18 mg. on the following day, representing in all only 18 % of the dose. In the dog the same dose would have yielded a rise in neutral sulphur and ethereal sulphate, corresponding respectively to 47 % and 25 % of the dose. Taking the two sulphur fractions together, 18 % in the pig should be compared with 72 % in the dog [Coombs and Hele, 1926]. On the dose-day 1125 mg. iodine (44 % of the dose) was excreted and on the following day 369 mg. (14.5 % of the dose), showing that there had been no failure in absorption. We repeated

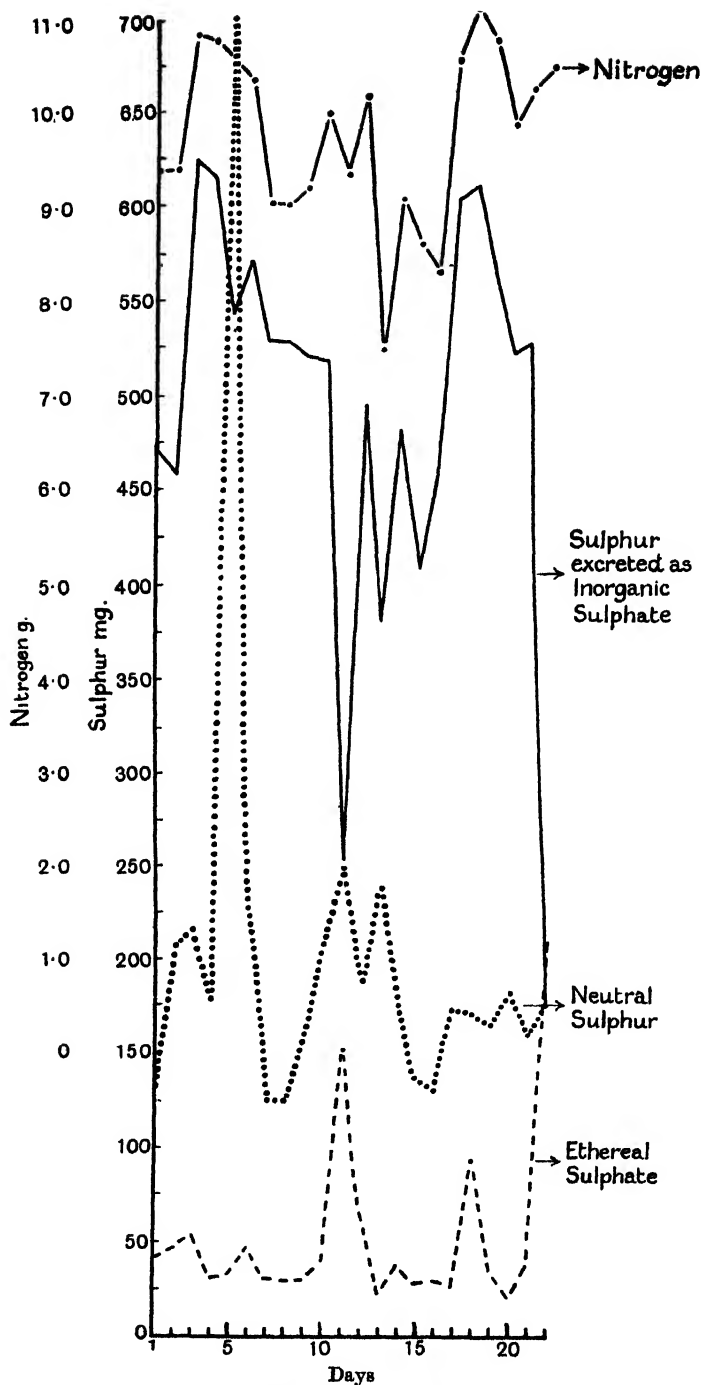


Fig. 2. "Rosamund."

Day No. 5. 6.36 g. ( $2 \times \text{m.w./100 g.}$ ) *p*-bromophenylmercapturic acid.

„ No. 11. 6.28 g. ( $4 \times \text{m.w./100 g.}$ ) bromobenzene.

„ No. 18. 4.4 g. ( $2 \times \text{m.w./100 g.}$ ) *p*-iodophenol.

„ No. 22. 6.12 g. ( $3 \times \text{m.w./100 g.}$ ) iodobenzene.

This figure may be compared directly with the figures in Papers III, IV and V of this series. The figure in Paper I is however drawn to a different scale.

the experiment with a larger dose (6.12 g.) on the second pig (Fig. 2, day 22) and obtained exactly similar results.

We administered 3.14 g. bromobenzene to the first pig (Fig. 1, day 21) and obtained a small rise in the output of neutral sulphur, equivalent to 6.7 % of the dose. This increase might be attributed to normal variations in metabolism. The rise in ethereal sulphate corresponded to 14 % of the dose. A larger dose (6.28 g.) was given to the second pig (Fig. 2, day 11). The rise in ethereal sulphate on the dose-day indicated an excretion of 10 % of the dose and on the following day 3 %. The effect on the neutral sulphur is not so easy of interpretation. The neutral sulphur had risen to 212 mg. on the previous day. The actual increase over this figure on the dose-day was only 59 mg. S (4.6 % of the dose). This was followed by a fall and a subsequent rise. We regarded these variations as extraneous in origin and without relation to the administration of the bromobenzene. In the dog the rise in neutral sulphur would have been about 600 mg. S. However, the fact that a small rise in neutral sulphur did occur in the pig after bromobenzene made us attempt the isolation of a mercapturic acid from bromobenzene and also from iodobenzene. Although the usual precautions were taken, we failed to isolate any. The results are summarised in Table II.

Table II. *The effect of the monohalogenbenzenes on metabolism.*

Percentage of dose excreted as:

	Ethereal sulphate		Neutral sulphur		Halogen	
	Pig	Dog	Pig	Dog	Pig	Dog
Iodobenzene	%	%	%	%	%	%
	18	25	Nil	47	58.5	79
Bromobenzene	19	30	Nil	47	51	68
	14	28	6.7 (?)	47.2	—	—
Chlorobenzene	13	25	4.6 (?)	62.6	—	—
	—	24.9	—	34.8	—	57.9
	—	19.2	—	35.8	—	55.2

The numbers for the dog are taken from Papers II, III and IV of this series.

*The effect of the mercapturic acids on metabolism.*

To the first pig (Fig. 1, day 32) we administered *p*-iodophenylmercapturic acid. The rise in the neutral sulphur output on the first day corresponded to 83 % and the iodine excretion to 76 % of the dose. There was no appreciable rise in the ethereal sulphate. The excretion of the mercapturic acid was over within 24 hours. There was no haemoglobinuria.

As a control an equivalent dose of *p*-iodophenylmercapturic acid was given to a large female dog. The pig, weighing 18 kg. received 3.65 g., the dog, weighing 11 kg., received 2.23 g. The dog's urine was free from haemoglobin for the first 24 hours following the dose. For the next 23 hours the urine contained haemoglobin but on the third day the urine was clear. On the first day the iodine estimations in the urine showed an excretion of only 9 % of the dose, on the second day 12 %. The haemoglobinuria showed exactly

the same features as those observed following the administration of the bromo- and chloro-compounds [Callow and Hele, 1927]. It is interesting to compare the rapid excretion of mercapturic acid by the pig and the slow excretion by the dog.

To the second pig (Fig. 2, day 5) 6.36 g. *p*-bromophenylmercapturic acid, or twice the equivalent dose of the *p*-iodophenylmercapturic acid used for the first pig, were given. The rise in the neutral sulphur indicated that 80 % of the dose was excreted in the first 24 hours and 6 % on the following day. None was converted to ethereal sulphate. There was no haemoglobinuria. If the dosage, g. per kg. of body-weight, is compared, this dose is nearly three times the amount which produced haemoglobinuria in the dog [Callow and Hele, 1927]. More than half of the mercapturic acid administered was recovered unchanged from the urine. The results are summarised in Table III.

Table III. *The effect of the mercapturic acids on metabolism.*

Percentage of dose excreted as:

	Ethereal sulphate		Neutral sulphur		Halogen	
	Pig %	Dog %	Pig %	Dog %	Pig %	Dog %
<i>p</i> -Iodophenylmercapturic acid	Nil	Nil	83	(?)	76	21
<i>p</i> -Bromophenylmercapturic acid	Nil	Nil	86	48	—	—
<i>p</i> -Chlorophenylmercapturic acid	—	Nil	—	(?)	—	36

The numbers for the dog are taken from this paper and from Paper V of this series.

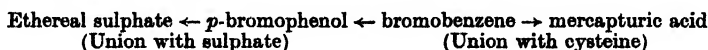
#### DISCUSSION.

From our results we feel justified in concluding that the metabolism of our pigs was very different from that of our dogs. The pig seems incapable of synthesising mercapturic acids. It is clear also that the pig does not synthesise ethereal sulphates with the same readiness as does the dog. In our experiments glycuronic acid was excreted and the increase in the reducing power of the urine, following the administration of phenolic compounds, suggested that the pigs detoxicated foreign substances of this sort mostly by the glycuronic acid synthesis, but there is no exact proof. It is to be noted that in our experiments with one exception (*p*-iodophenol, Fig. 2, day 18) the rise in ethereal sulphate was accompanied by a fall in inorganic sulphate. We know that the dog can synthesise ethereal sulphate directly from at least one phenol and sulphate [Hele, 1924, 1] and we have no reason to believe that the pig cannot do the same. However, we regard the conclusive proof of this in the pig as extremely difficult or impossible, because the pig does not synthesise ethereal sulphates readily.

Shiple, Muldoon and Sherwin [1924] express the view that there are two ways of detoxicating phenolic substances, the one by union with some intermediary sulphur compound of endogenous metabolism, which is oxidised to ethereal sulphate, and the other by utilising exogenous cystine to form a mercapturic acid or like substance, which may be either excreted unchanged or oxidised to ethereal sulphate wholly or in part. In the pig they obtained

a rise in neutral sulphur after the halogen-benzenes but were apparently unable to isolate a mercapturic acid from that animal [Muldoon, Shiple and Sherwin, 1923]. On the other hand, they believe that ethereal sulphates cannot be formed by the direct union of phenols with preformed sulphate, whether arising endogenously or exogenously. Their views are based on work on two animals, a dog of 15.5 kg. [Muldoon, Shiple and Sherwin, 1924] and a pig of 24 kg. [Shiple, Muldoon and Sherwin, 1924]. Their ideas are in accordance with theories put forward by Thomas and Straczewski, Zeller and Straczewski, Kapfhammer and Rhode, theories already discussed in this series [Hele, 1924, 2]. It is not clear however that the experimental evidence adduced by the American workers will bear none other than the interpretation put upon it by the authors.

In the work on the dog [Muldoon, Shiple and Sherwin, 1924, p. 679] the only suggestion of a controversial kind is the theory put forward that ethereal sulphate is solely formed from endogenous sulphur. The experimental basis for this theory is the fall in output of ethereal sulphate when cystine is given to a dog receiving a daily dose of bromobenzene. There does not seem, however, conclusive proof that such a fall occurs under these circumstances. The authors consider mercapturic acid to be derived from the union of bromobenzene with exogenous sulphur and ethereal sulphate from the union of bromobenzene with endogenous sulphur. Any increase therefore of exogenous sulphur would tend to upset the balance, increasing the mercapturic acid at the expense of the ethereal sulphate. They assume as others have done that the intermediary in either case is a phenol. We have however shown [Coombs and Hele, 1926] that the phenols are not intermediaries in the synthesis of mercapturic acids. When the series of reactions



is considered, it is clear that the decrease in ethereal sulphate, if it really does occur, must be due to an increased formation of mercapturic acid as the concentration of available cysteine is raised. There is no necessity to distinguish between endogenous and exogenous metabolism. In the second paper of this series [Hele, 1924, 2] stress was laid on the uniformity of the response of dogs to the halogen-benzenes, but some evidence, not very conclusive, was put forward in that paper to prove that the balance could be upset, if dosage with sulphate or with cystine was continued for more than one day.

The experimental work on the pig summarised in the first table of the American authors (p. 61) is not antagonistic to our experimental results and our views except in one particular. They found an increase in ethereal sulphate following the administration of *p*-bromophenylmercapturic acid. Perhaps it may be allowed that animals of the same species may vary in their power to carry out certain oxidations. We find it, however, very difficult from structural considerations to conceive of any oxidation of a mercapturic acid to ethereal sulphate without the fission of the molecule and the liberation of

a phenol, which might then be detoxicated by direct union with sulphate. This suggestion has already been mooted in the second paper of this series [Hele, 1924, 2]. An oxidation of a mercapturic acid to a sulphonic acid, the sulphur of which would still appear in the neutral sulphur fraction, is much more likely to occur [König, 1892]. In the rest of the table evidence is collected, which shows that the oral administration of cystine assists in the formation of ethereal sulphate from phenols, while sodium sulphate does not. We would expect cystine to be the more efficacious, because it is excreted more slowly, being in an unoxidised form, and because very likely it is oxidised at the seat of ethereal sulphate synthesis, thus producing sulphate where it is most required [Hele, 1924, 1]. For reasons already given we feel it would be very difficult to obtain conclusive proof of the formation of ethereal sulphate in the pig from preformed sulphate, even if the nitrogen-free diet, utilised by the American workers, were employed. Contrary to their findings we obtained generally a fall in inorganic sulphate when the ethereal sulphate rose. The pig behaves in this respect like the dog.

In the second table (p. 62) of the American work on the pig there are several findings contradictory to our theories. Bromobenzene caused a rise in neutral sulphur, and added cystine caused a further rise with a decrease in the output of ethereal sulphate. The effects of *p*-chlorophenol and cystine were similar. The fall in ethereal sulphate, which is not very great, may have been accidental and part of the neutral sulphur may be attributed to failure in oxidation of the cystine apart from any effect of the derivatives of benzene. This is certainly true in the *p*-chlorophenol—cystine experiments. The rise in neutral sulphur is about 400 mg. S. The dose (0.6 g.) of *p*-chlorophenol given could not theoretically have given rise to more than 150 mg. S. In this table there is also further evidence of the oxidation of *p*-bromophenylmercapturic acid.

In the American work on the pig there are only two findings which are seriously antagonistic to our theories; the rise in neutral sulphur after bromobenzene and the similar rise after *p*-chlorophenol. We believe that phenols bear no relation to the formation of mercapturic acids [Coombs and Hele, 1926] and even if these findings of the American workers are incontestable, it does not follow that such a rise in neutral sulphur indicates the formation of a mercapturic acid. It might be due to a sulphonic acid.

There are two defects in the work of the American authors, which are not unimportant. The variations in the output of nitrogen are considerable. These variations cannot always be attributed to the nature of the substances administered. Such variations indicate variations also in the metabolism of tissue sulphur, variations which would affect the neutral sulphur of the urine. It is true that these variations are most marked in the first "pig" table, where they have but little bearing on the authors' argument and it is probable that such variations are bound to occur when a nitrogen-free diet is employed, or at any rate are more fully exposed to view when the nitrogen excretion is

low. The second experiment described in this paper is open to the same criticism in a lesser degree. We have however taken into consideration these variations in nitrogen output and we do not think that our conclusions are inadmissible.

The second defect in the American work is avoidable. We have always found it necessary to allow a considerable interval of time to elapse between each stage of an experiment, in order to make certain that the metabolism has again reached the normal. It is true that in our first "pig" experiment we administered phenol almost immediately after guaiacol carbonate. In this particular experiment however we were testing merely the general effect of phenolic substances. The administration of guaiacol and phenol constituted one experiment. We have made an approximate division in the output of ethereal sulphate between these substances in the table as an indication of a general thesis. The American workers, however, were carrying out many different tests, and in our opinion they did not leave sufficient intervals of time between each stage of their enquiry. It is not always clear to what cause to attribute a particular effect.

In our experiments we have found that mercapturic acids produce haemoglobinuria in the dog, an animal capable of synthesising these compounds. On the other hand, we have found that the pig cannot synthesise mercapturic acids and that mercapturic acids in much larger doses per kg. of body-weight have no effect on the pig. The American workers also do not describe any toxic effects following the administration of mercapturic acids to the pig and the same seems true for the rabbit [Rose, Shipley and Sherwin, 1924]. Perhaps, too, the rabbit is incapable of synthesising mercapturic acids [Muldoon, Shipley and Sherwin, 1923]. This difference indeed seems remarkable, although it is possible that mercapturic acids in massive doses might cause haemoglobinuria even in the pig. The rapid excretion of mercapturic acids by the pig may be attributed to the fact that the kidney of the pig is not in any way blocked by red cell debris, the result of haemolysis.

#### SUMMARY.

1. The pig, unlike the dog, does not readily synthesise ethereal sulphate. For this reason it would be extremely difficult, if not impossible, to obtain conclusive evidence of the direct formation of ethereal sulphate from phenolic compounds and preformed sulphate. On the other hand, there is no evidence that the pig does not carry out this direct synthesis in the same manner as the dog.
2. The pig unlike the dog is probably unable to synthesise mercapturic acids.
3. The pig like the dog cannot convert mercapturic acids to ethereal sulphates.
4. Mercapturic acids in doses three times the toxic dose for the dog per kg. of body-weight do not produce haemoglobinuria in the pig.



The authors desire to thank the Government Grant Committee of the Royal Society for grants in aid of this research and one of them (H. I. C.) the Medical Research Council for a grant.

## REFERENCES.

- Brazier and McCombie (1912). *J. Chem. Soc.* **101**, 972.  
(3) Callow and Hele (1926). *Biochem. J.* **20**, 598.  
(5) ——— (1927). *Biochem. J.* **21**, 606.  
(4) Coombs and Hele (1926). *Biochem. J.* **20**, 606.  
Folin and Denis (1915). *J. Biol. Chem.* **22**, 305.  
——— (1916). *J. Biol. Chem.* **26**, 507.  
(1) Hele (1924, 1). *Biochem. J.* **18**, 110.  
(2) ——— (1924, 2). *Biochem. J.* **18**, 586.  
Kendall (1920). *J. Biol. Chem.* **43**, 149.  
König (1892). *Z. physiol. Chem.* **16**, 525.  
Muldoon, Shiple and Sherwin (1923). *Proc. Soc. Exp. Biol. Med.* **21**, 145.  
——— (1924). *J. Biol. Chem.* **59**, 675.  
Rose, Shiple and Sherwin (1924). *Amer. J. Physiol.* **69**, 518.  
Shiple, Muldoon and Sherwin (1924). *J. Biol. Chem.* **60**, 59.

# LXXXVI. STUDIES IN THE SULPHUR METABOLISM OF THE DOG.

## VII. THE EFFECT OF FLUOROBENZENE ON SULPHUR METABOLISM.

By HERBERT ISAAC COOMBS.

*From the Biochemical Laboratory, Cambridge.*

*(Received May 2nd, 1927.)*

IN a former communication of this series [Coombs and Hele, 1926] it was shown that very small differences in chemical constitution very much alter the metabolism of the halogen-benzenes (chloro-, bromo- and iodo-benzenes). These latter bodies all give rise to a mercapturic acid in the urine of dogs after they have been ingested. They are, however, the only compounds which have been definitely proved to cause the synthesis—the substitution of even such a group as methoxyl in the ring causing the metabolism to take a different course. Evidence has been obtained, however, that the *o*- and *m*-dichlorobenzenes and benzene itself probably also give rise to mercapturic acids [Callow and Hele, 1926].

Perhaps the most likely compound which might be expected to form a mercapturic acid is the remaining halogen-benzene, fluorobenzene. This compound fits exactly into the series with the other halogen-benzenes in all its chemical and physical properties, and there would therefore seem no reason why it should not also have a similar effect on sulphur metabolism. To test this point a quantity of fluorobenzene was made and administered to dogs with the results shown in the figures. Benzene itself was also administered with the result shown.

### EXPERIMENTAL.

About 150 g. fluorobenzene were prepared by the method of Holleman [1904]. The method gave a very pure product but not in very good yield; the only difficulty that was encountered was the solvent action of the mercury of the mercury seal on the solder of the reaction vessel, but this was easily remedied by the substitution of a seal turned from one piece of copper.

The diet of the dog "Vixen" has been given in a former paper of this series and that of "Pansy" was as follows<sup>1</sup>:

		T.N.	T.S.	Calories
"Pansy," 8.6 kg.:		g.	g.	K
Meat	80 g.	2.73	0.181	106
Sugar	70 g.	—	—	286
Margarin	20 g.	—	—	156
Milk	180 cc.	0.83	0.074	115
		3.56	0.255	663

<sup>1</sup> This diet is similar to that given for "Pansy" in Paper V, but differs in sugar content.

It will be seen from the figures that there is a small but distinct rise in the neutral sulphur output following the administration of the fluorobenzene—amounting to about 8 to 11 % of the dose. This rise is small compared to the rise after the other halogen compounds where it is about 47 % [Coombs and Hele, 1926], and is more in accord with that caused by benzene. It must be remembered, however, that there is a great difference in the boiling points—fluorobenzene boiling at  $85^{\circ}$  whereas chlorobenzene boils at  $132^{\circ}$ . The disparity in the yield from these two compounds must be attributed, in part at least, to this volatility, because shortly after the fluorobenzene has been ingested it can easily be detected by its odour in the breath of the animal.

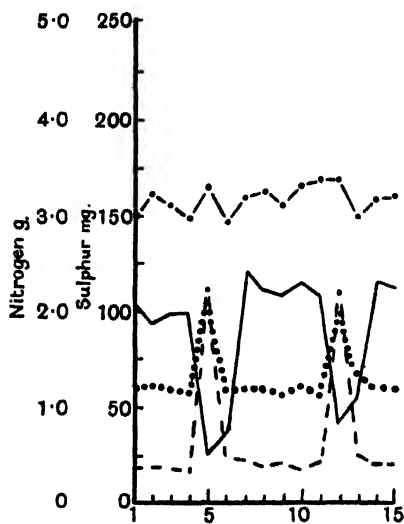


Fig. 1.

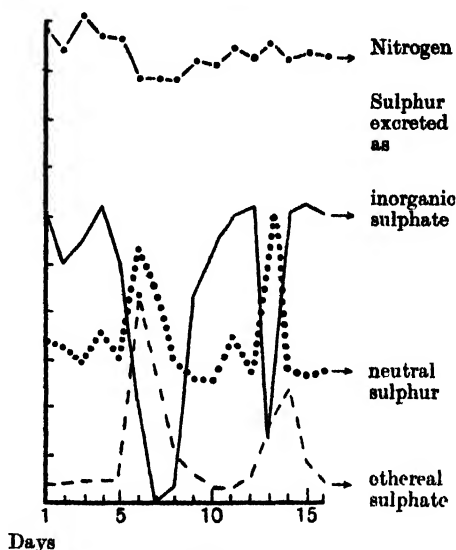


Fig. 2.

Fig. 1. "Pansy." Day No. 5.  $2 \times \text{m.w./100 g. fluorobenzene}$  } by mouth.  
 „ No. 12.  $2 \times \text{m.w./100 g. fluorobenzene}$  }

Fig. 2. "Vixen." Day No. 6.  $2 \times \text{m.w./100 g. fluorobenzene}$  } subcutaneously.  
 „ No. 13.  $2 \times \text{m.w./100 g. benzene}$  }

The author's best thanks are due to Dr T. S. Hele for his interest in this work, and the author also wishes to acknowledge gratefully grants from the Royal Society and the Medical Research Council.

#### REFERENCES.

- Callow and Hele (1926). *Biochem. J.* **20**, 598.  
 Coombs and Hele (1926). *Biochem. J.* **20**, 606.  
 Holleman (1904). *Rec. Trav. Chim. Pays-Bas*, **23**, 225.

## LXXXVII. THE REDUCTION OF HAEMATIN AND METHAEMOGLOBIN.

By ROBERT HILL  
AND HENRY FRANCIS HOLDEN  
(*Beit Memorial Research Fellow*).

*From the Biochemical Laboratory, Cambridge.*

(Received April 26th, 1927.)

THE question of the oxygen content of methaemoglobin relative to haemoglobin has long interested physiologists. The recent work of Keilin [1926] has brought haemochromogen into prominence as a type of important biological substances. The great ease with which haematin is reduced in comparison with protoporphyrin has led to the assumption that in the reduction of the former to reduced haematin the essential change is that of the valency of the iron. This would involve one equivalent of hydrogen per atom of iron. Küster [1910] suggested that methaemoglobin was a compound of haematin in which the iron was in the ferric state. In oxyhaemoglobin, haemoglobin, haemochromogen and reduced haematin the iron might therefore be held to be in the ferrous state. If this is the case, the term "oxygen content" of methaemoglobin and haematin has little meaning, as is pointed out by Conant and Scott [1926]. Determinations of the equivalents of hydrogen necessary for the reduction of haematin have failed to confirm this view. The data and conclusions of many workers are summarised by Nicloux and Roche [1926]. As a result of their own experiments these workers concluded that the reduction of methaemoglobin required two equivalents of hydrogen. Later, Roche [1926] contended that haematin needs four equivalents for its reduction. Their work has been discussed by Conant and Scott [1926].

Conant [1923] had previously titrated electrometrically both methaemoglobin and haematin and found one equivalent of hydrogen necessary for the former but two for the latter substance. He, however, considered the method unsuitable for haematin.

Haurowitz [1927] had reduced haematin with hydrazine hydrate and by measuring the nitrogen evolved found one equivalent for the reduction. It must however be taken for granted that the volume of nitrogen measured corresponds with the whole of the hydrogen used in reducing the pigment, for which the available evidence is satisfactory. For the reduction of haematin to reduced haematin, hydrazine hydrate is unsuitable because in itself it is a nitrogen compound capable of yielding a haemochromogen [Anson and Mirsky, 1925]. Haurowitz does not seem to have realised any difference between reduced haematin and haemochromogen.

Hill and Holden [1926] prepared methaemoglobin by direct combination of haematin and globin. In view of the discrepancies in the above results it was decided to attempt to titrate directly with a reducing agent solutions of methaemoglobin, of haemin in alkali, and of haemin in alkali in the presence of pyridine.

The essential requirement of such a research was that it should be simple and direct both in theory and technique.

The choice of a suitable reducing agent was the first consideration. From the limited number of available reducers ferrous tartrate in alkaline solution was selected. In practice, standard ferrous sulphate solution was added to an alkaline solution of the pigment containing sodium tartrate. By this means errors due to atmospheric oxidation of the standard reducing agent itself were reduced to a negligible amount. The only state to which a ferrous salt is readily oxidised is that of a ferric salt. The reduction potential of the simpler ferrous compounds increases greatly on passing from an acid to an alkaline reaction. Excess of ferric tartrate (the product of its oxidation) was found to be without appreciable influence. No additional standardisation is necessary if pure ferrous ammonium sulphate is used. The possibility of the reaction stopping at the formation of ferrosferric compounds is negated by the indifference of excess of ferric tartrate.

As ordinarily prepared, methaemoglobin contains undesirable amounts of ferricyanide which are not readily removable by dialysis. Nicloux's method gives a 60 % conversion of oxyhaemoglobin to methaemoglobin, but such a solution was unsuitable for these experiments. The preparation of a sufficient quantity of pure globin was too tedious owing to its high equivalent weight. Accordingly advantage was taken of the fact that at an acid reaction at which oxyhaemoglobin is still stable ferric tartrate oxidises it completely to methaemoglobin. If such a solution be immediately made alkaline the ferrous salt formed reduces some of the methaemoglobin. It was found possible to remove the ferrous salt and the bulk of the ferric salt by suitable treatment without such reduction occurring.

In the case of haematin, since both reduced haematin and pyridine haemochromogen are insoluble, they can be separated from the soluble haematin by centrifuging. The end-point can thus be determined.

In the case of methaemoglobin the oxyhaemoglobin produced by a known amount of reducer followed by oxygen was estimated by determining the oxygen capacity of the solution.

#### *The preparation of methaemoglobin.*

1 litre of fresh defibrinated ox blood was centrifuged and the corpuscles washed three times with 0.9 % sodium chloride solution. To 500 cc. of corpuscles so obtained were added 4.5 l. of water, 500 cc. 25 % sodium tartrate (Rochelle salt is not suitable) and 250 cc. 10 % acetic acid by volume. After

thorough mixing, 60 g. sodium tartrate and 40 g. iron ammonium alum dissolved in 500 cc. of cold water were slowly added with stirring. Bubbles were given off in the liquid and the methaemoglobin spectrum rapidly replaced that of oxyhaemoglobin. After standing the turbid liquid was filtered from the stroma, etc., the clear filtrate was diluted with twice its volume of water and allowed to stand overnight at 0°. The methaemoglobin was practically completely precipitated as a granular precipitate. This precipitate contained ferric tartrate and was completely insoluble in distilled water. It was soluble in very dilute alkalis yielding first a clear neutral solution of methaemoglobin, from which the iron could be dialysed. That the precipitating agent was ferric and not ferrous tartrate could be shown as follows. On suspending the washed precipitate in distilled water and adding a small amount of sodium hydro-sulphite, as reduction proceeded the precipitate dissolved, finally yielding a perfectly clear solution of haemoglobin from which oxyhaemoglobin was obtained. The granular precipitate of methaemoglobin was washed with 2 l. of distilled water containing 2 cc. 10 % acetic acid and 20 cc. 25 % sodium tartrate. After expressing the superfluous liquid the precipitate was ground into a cream with water and dissolved with the minimum amount of 10 % sodium carbonate, added slowly from a burette, about 100 cc. being required. To the 620 cc. of solution so obtained were added 5 cc. of 25 % sodium tartrate and enough saturated ammonium sulphate to make a total volume of 1 l. The solution was then filtered. To the filtrate 1.5 l. saturated ammonium sulphate were added and, after standing overnight at 0°, the precipitate of methaemoglobin was filtered off. The iron remained in the filtrate. After washing with saturated ammonium sulphate the precipitate was pressed as dry as possible between filter papers and dissolved in half its weight of distilled water. This strong methaemoglobin solution was used directly for the experiments. After complete reduction of a measured sample with ferrous tartrate its oxygen capacity was determined by Barcroft's method. The tint of the oxyhaemoglobin of this sample was compared at known dilution with diluted defibrinated blood of known oxygen capacity. They were found to agree, showing that all the haematin present was in the form of methaemoglobin. Hence the precipitation of methaemoglobin by ferric tartrate under the conditions of the experiment does not alter the essential properties of the conjugated protein. When freed from the iron tartrate the oxyhaemoglobin finally obtained resembles in solubility, oxygen capacity, and colour the original oxyhaemoglobin.

#### *The reduction of haematin.*

The experiments were conducted in small centrifuge tubes about 62 × 18 mm. The haematin solution was made by dissolving a known weight of about 0.3 g. in 25 cc. of dilute alkali. It was used immediately. Recrystallised ferrous ammonium sulphate was dissolved in water containing a trace of sulphuric acid to give a 0.02 *N* solution. A suitable amount of an alkaline tartrate

solution was used. A layer of toluene prevented reoxidation. To a series of four tubes were added equal amounts of haematin, alkaline tartrate solution and toluene, and an amount of water to make the total volume after addition of the reducer up to 8 cc. Four different volumes of the ferrous sulphate solution were added to the different tubes. They were stirred gently and immediately centrifuged after cautious removal of the stirrers. After 5 minutes the tubes were removed from the centrifuge and observed. In Exp. 1 they were observed in sunlight without centrifuging. The details of two typical experiments are recorded and the results for a series given in Table I.

Table I.

For 2 cc. haematin titrated with standard ferrous iron solution as reducer.

Remarks	cc. reducer added to effect reduction	Observed correction in cc. of reducer for dissolved oxygen	cc. reducer used in reducing haematin	Theoretical cc. reducer for 1 equivalent	Equivalent
Pyridine haemochromogen in sodium carbonate	2.40	0.35	2.05	2.31	0.89
Same, with 1.5 cc. <i>N</i> /5 ferric iron	2.30	0.35	1.95	2.31	0.84
Reduced haematin in sodium hydroxide	2.10	0.30	1.80	1.81	0.99
Pyridine haemochromogen in sodium hydroxide	2.30	0.35	1.95	1.95	1.00
Pyridine haemochromogen in sodium hydroxide	2.20	0.35	1.85	1.95	0.95

*Exp. 1.* The alkaline tartrate is a mixture of 20 cc. 10 %  $\text{Na}_2\text{CO}_3$  + 10 cc. 25 % sodium tartrate. Ferrous ammonium sulphate, 0.4015 g. in 50 cc. Haemin 0.3329 g. in 25 cc. containing 0.2 cc. purified pyridine. Each tube contained 2 cc. haematin solution + 2 cc. alkaline tartrate + 1 cc. toluene and water to make 9 cc. total volume.

Tube	Ferrous sulphate cc.	Observation
1	2.3	Nearly reduced
2	2.5	Reduced
3	2.7	"
4	4.0	"

Zero correction for dissolved oxygen using very dilute haematin.

1	0.1	Not reduced
2	0.2	"
3	0.3	"
4	0.4	Reduced

*Result.* 2 cc. of 0.0237 *N* haematin required 2.40–0.35 cc. of 0.0205 *N* iron.

*Exp. 2.* The alkaline tartrate is a mixture of 1 cc. 25 % sodium tartrate, 1 cc. 40 % sodium hydroxide and 1 cc. of water. Ferrous ammonium sulphate; 0.8073 g. in 100 cc. Haemin; 0.6054 g. to 50 cc. Each tube contained 2 cc. haematin solution + 2 cc. alkaline tartrate + 2 cc. toluene + water to make 9 cc. total volume.

Tube	Ferrous sulphate cc.	Observation
1	1.8	Not reduced
2	2.0	"
3	2.2	Reduced
4	2.5	"
Zero correction using very dilute haematin.		
1	0.1	Not reduced
2	0.2	"
3	0.3	Just reduced
4	0.4	Reduced.

*Result.* 2 cc. of 0.0186 *N* haematin required 2.1–0.30 cc. 0.0206 *N* iron.

*The reduction of methaemoglobin.*

5 cc. of a strong solution of methaemoglobin prepared as described above were measured into a test-tube. To this was added 0.7 cc. of a solution prepared by mixing 50 cc. 25 % tartrate, 36 cc. water and 14 cc. of strong ammonia solution (Sp. Gr. 0.880). A measured quantity of water was added so that, with the volume of the reducing agent to be added subsequently, the total volume of liquid would be 8.5 cc. A stirrer was then placed in the tube and a layer of liquid paraffin 1 cm. deep carefully poured on. The ferrous ammonium sulphate solution (containing 0.2023 g. of the salt in 25 cc. of water) was run in from a burette with a long drawn-out jet reaching nearly to the bottom of the tube. The stirring was continued for 1 minute after the reducer had been added and the mixture allowed to stand 15 minutes. The solution of haemoglobin was then drawn up in a pipette from beneath the paraffin and transferred to a conical flask where it was gently shaken for 15 minutes with oxygen from a cylinder. 3 cc. of the resulting solution of oxyhaemoglobin were measured out into the bulbs of two Barcroft differential gas apparatus. The oxygen capacity was then directly determined by the addition of ferricyanide. The results of the experiments are set forth in Table II.

Table II. *Reduction of methaemoglobin as measured by oxygen capacity with standard ferrous iron solution.*

cc. reducer added	Observed oxygen capacity cc.	Calculated oxygen capacity for 1 equivalent cc.	Calculated oxygen capacity for 2 equivalents cc.	Observed equivalent
2.35	{ 838 848	948	474	{ 1.11 1.10
2.30	{ 806 788	920	460	{ 1.12 1.15
2.30	{ 781 751	920	460	{ 1.16 1.20

A similar series of experiments was now made using a Haldane evolution apparatus. Different amounts of reducer were added and the corresponding oxygen capacities determined by adding ferricyanide. The zero correction for



dissolved oxygen was determined as in the case of haematin, using a very weak solution of methaemoglobin. The results of this series are shown in Fig. 1. The ordinates represent the oxygen capacity in cc. in terms of 5 cc. of the original methaemoglobin solution, the abscissae represent cc. of a standard iron solution (0.02 *N*) added. The dotted curves represent the theoretical oxygen capacities for the reduction involving one and two equivalents of iron per molecule of methaemoglobin.

#### DISCUSSION OF RESULTS.

The high equivalent weights of haemin (651) and of methaemoglobin (16,700) militate against the experimental accuracy with which the addition to either of one equivalent of hydrogen can be determined. In addition, both substances are known to undergo certain changes in solution. The reaction between reducing agents and such decomposition products is as yet obscure. Such decomposition might be expected to affect the two methods of estimation employed in opposite directions decreasing the amount of oxygen obtainable

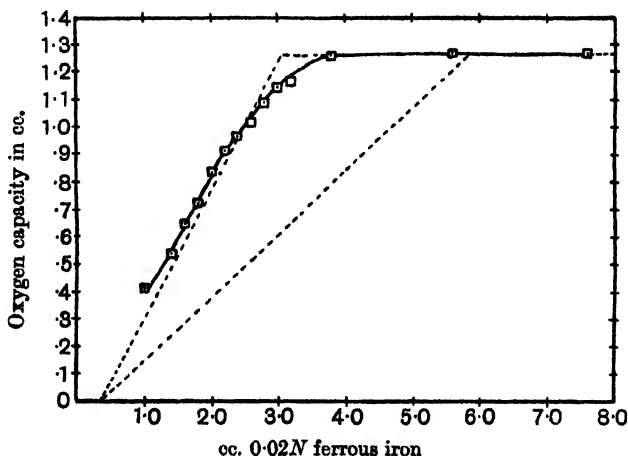


Fig. 1.

from the conjugated protein after treatment with a given amount of reducer and decreasing the amount of reducer required for a known volume of haematin solution. The mean value for the case of haemochromogen and reduced haematin is 93 % of the theoretical value for one equivalent of hydrogen. Of the two samples of haemin used the more recently prepared one gave the values most nearly approaching to the theoretical value. The determinations of the resulting oxygen capacity on reduction of a single specimen of methaemoglobin showed a progressive decrease from day to day. The mean value of four determinations carried out within 2 days after the preparation of the methaemoglobin gave a ratio of equivalents of hydrogen/molecular oxygen of 1.12. The effect of reducible impurities in the methaemoglobin is clearly shown by the curve. If the methaemoglobin were perfectly pure, a sharp break in the

curve would be expected to occur when the theoretical amount of reducer had been added. The curve, however, tails off gradually to a constant oxygen capacity corresponding to complete reduction. The methaemoglobin, however, is completely reduced long before two equivalents of reducer have been added, and for smaller amounts of reducer closely follows the slope of the theoretical curve for one equivalent.

It would seem probable that the change of methaemoglobin to haemoglobin or of haematin to either reduced haematin or haemochromogen is one and the same, namely, the addition of one equivalent of hydrogen per atom of iron. The protein would appear to play no essential part in this reduction and does not react with the reducing agent. Methaemoglobin is a compound of haematin and globin while haemoglobin is a compound of reduced haematin and globin. Küster's hypothesis [1910] is thus experimentally confirmed.

#### SUMMARY.

1. Methaemoglobin has been prepared by the use of ferric tartrate instead of potassium ferricyanide.
2. The protein can be completely precipitated by ferric tartrate without denaturation.
3. The reduction of methaemoglobin to haemoglobin requires one equivalent of hydrogen per atom of iron.
4. The reduction of haematin to reduced haematin or to pyridine haemochromogen requires one equivalent of hydrogen per atom of iron.

The authors desire to record their gratitude to Sir F. G. Hopkins for his advice and encouragement. One of them (R. H.) is indebted to the Medical Research Council for a grant.

#### REFERENCES.

- Anson and Mirsky (1925). *J. Physiol.* **60**, 50.  
 Conant (1923). *J. Biol. Chem.* **57**, 401.  
 Conant and Scott (1926). *J. Biol. Chem.* **69**, 575.  
 Haurowitz (1927). *Z. physiol. Chem.* **66**, 249.  
 Hill and Holden (1926). *Biochem. J.* **20**, 1326.  
 Keilin (1926). *Proc. Roy. Soc. Lond. B.* **94**, 266.  
 Küster (1910). *Z. physiol. Chem.* **66**, 249.  
 Nieloux and Roche (1926). *Bull. Soc. Chim. Biol.* **8**, 71.  
 Roche (1926). *Bull. Soc. Chim. Biol.* **8**, 362.

# LXXXVIII. THE PHYSIOLOGICAL RÔLE OF VITAMIN B. PART III. STUDY OF VITAMIN B DEFICIENCY IN PIGEONS<sup>1</sup>.

BY STANISLAW KAZIMIERZ KON  
AND JACK CECIL DRUMMOND.

*From the Department of Physiology and Biochemistry, University  
College, London, and the State School of Hygiene, Warsaw.*

*(Received March 23rd, 1927.)*

THE recognition that there exists a close relationship between the consumption of food and the supply of vitamin B in the diet has greatly advanced attempts to determine the physiological rôle of this substance. It has, in the first place, provided a plausible explanation of many of the numerous divergencies of opinion that are recorded in the literature, whilst secondly it has, we believe, indicated the right line along which future research in this field should proceed.

It is obvious from a survey of the literature that the great majority of investigators have disregarded entirely or failed to appreciate fully the significance of the voluntary abstention from food that animals deprived of vitamin B usually exhibit. A few have borne this in mind, notably Funk [1924, p. 196].

## OBJECT OF THE EXPERIMENTS.

In view of the conclusions reached by Drummond and Marrian [1926] concerning the part played by inanition in vitamin B deficiency in rats it seemed imperative that similar experiments should be carried out on pigeons. It was therefore planned to study the fate of pigeons fed on dietaries lacking vitamin B, but under conditions where the effects of inanition could be separated from those due more directly to the deficiency.

It is obvious that the experimental study of vitamin deficiency can give clear-cut results only when the vitamin under investigation is the sole limiting factor of a diet complete with regard to all other components and when all factors liable to affect the experiment are checked by the use of proper controls. In the present paper a new method of studying vitamin B deficiency is described, as well as the results obtained on pigeons with the aid of this method, which it is believed provides a control covering the disturbing effects of inanition.

Not less than six experimental animals are placed on the deficient diet. For each of them a control of the same weight and if possible the same age and sex is selected. The control animals are given the same amounts of the diet

<sup>1</sup> Recent work has clearly demonstrated the existence of at least two substances of the vitamin type in the yeast extracts employed by us. The name vitamin B used throughout this paper covers both these factors and possibly others yet undiscovered.

as are eaten voluntarily by the corresponding animals fed on the deficient diet, but in addition receive a liberal supply of the missing factor. Both lots are thus placed under identical conditions, except for the vitamin factor. It is therefore reasonable to assume that differences of behaviour exhibited by the deficient group as compared with the controls are attributable to a lack of the vitamin<sup>1</sup>.

#### EXPERIMENTAL.

Pigeons were used as experimental animals, and were divided into four groups of eight birds, each kept in a separate cage. The individual initial weights (g.) of the pigeons are given in Table I.

Table I.

Lot I		Lot II		Lot III		Lot IV	
Pigeon	Weight	Pigeon	Weight	Pigeon	Weight	Pigeon	Weight
I a	414	II a	423	III a	337	IV a	353
I b	445	II b	443	III b	367	IV b	357
I c	445	II c	428	III c	398	IV c	400
I d	372	II d	380	III d	503	IV d	513
I e	457	II e	454	III e	443	IV e	447
I f	425	II f	433	III f	387	IV f	383
I g	395	II g	405	III g	411	IV g	410
I h	383	II h	355	III h	497	IV h	465
Average	417		415		418		416

Lot I received an artificial ration deficient in vitamin B. Each bird in Lot II received by the mouth the same quantity of food that the corresponding animal in Lot I had eaten in the previous 24 hours *plus* 1 g. of yeast extract (marmite). Lots III and IV were daily fed forcibly with amounts of the deficient ration calculated to maintain their weight if assimilated, Lot IV receiving in addition 1 g. of yeast extract every day.

*Diet.* A modified artificial diet of the type used by Randoin and Simonnet [1924, 1] was used. Polished rice, although much simpler to use, is by no means an adequate diet for the pigeon [McCollum and Davies, 1915; McCarrison, 1921], and in our opinion is unsatisfactory for use in such studies as these.

The ration had the following composition:

Rice starch	...	...	66 %	Butter fat	...	...	...	4 %
Caseinogen purified	...	...	16 %	Filter-paper pulp	...	...	...	2 %
Agar-agar	...	...	8 %	Salt-mixture (McCollum)	...	...	...	4 %

The foodstuffs were mixed with a sufficient quantity of water (4.5 litres for 6 kg. of the diet) to a stiff dough, the agar being added last, and placed in flat trays in a gas oven for 1-2 hours at 130°. The resulting loaves were cut in a bread-cutting machine into slices and then into cubes the size of small peas, and finally dried at 50-60°. The diet used for forcible feeding was not cut up, but the loaves were ground to a coarse powder after drying. The diet, when offered for voluntary consumption (Lot I), was placed in glass pots covered with a tin lid having a small hole in the middle permitting the pigeon

<sup>1</sup> After sending this paper to Press, we found that Gulick [1922] has described a similar technique in a short note published in the *Proceedings of the American Physiological Society*.

to pick up the diet without scattering. The quantities offered were always in excess of the daily voluntary intake and were replenished daily. For artificial feeding the powdered diet was mixed with a sufficient amount of water and placed by means of a glass syringe and a piece of rubber tubing in the pigeon's crop. The control lots (II and IV) were also given daily by means of a small funnel and a piece of rubber tubing 2.5 cc. of yeast extract solution (corresponding to 1 g. of marmite). All the birds were given once a fortnight five drops of cod-liver oil. The results obtained with Lots I and II, and III and IV will be dealt with separately.

#### LOTS I AND II.

Every day the birds were weighed and determinations of the food consumed by Lot I made. Each bird in Lot II received the same amount of food as consumed by the corresponding bird in Lot I during the previous 24 hours, together with the supplement of 1 g. of yeast extract in solution.

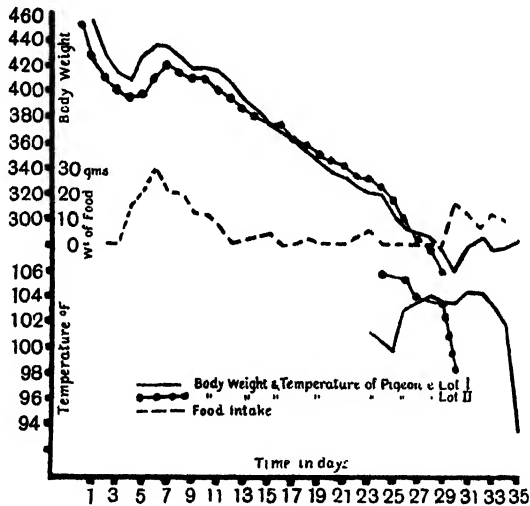


Fig. 1.

In Figs. 1, 2 and 3 are shown three typical records of body weights, food consumptions and, in the latter stages of the experiment, of body temperatures of birds in Lot I and their controls in Lot II. Fig. 4 shows the average curves for all birds in Lots I and II; this series covers a period of 28 days up to the death of pigeon *d* in Lot II. The average daily loss was 5.5 g. for Lot I and 5.0 g. for Lot II. The average daily food intake was 6.1 g. The average daily loss in complete starvation is about 9.3 g. (McCarrison). A general survey of the body weight and food intake curves demonstrates that the initial loss of weight is associated with insufficient food intake. In fact, during the first days of the experiment the pigeons, unaccustomed to be confined in individual cages, have no appetite. After a few days, however, they feed well and consume 20-30 g. of the diet, with corresponding rise in weight curves.

Later, lack of appetite becomes manifest in those deprived of vitamin B and from that time these pigeons are in a state of partial or even total starvation. In the beginning of the fourth week they sometimes regain partially their appetite and quantities of 10–15 g. of the diet may be voluntarily ingested. A possible explanation of this phenomenon will be discussed later.

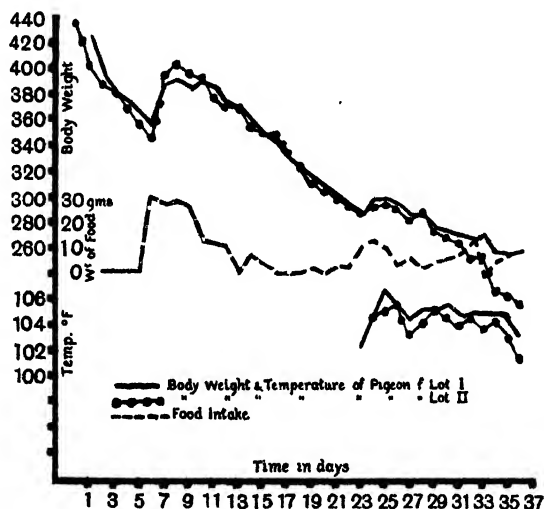


Fig. 2

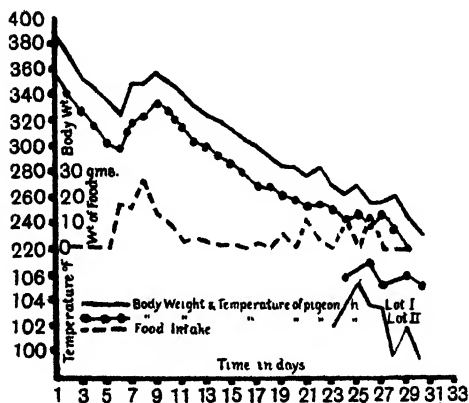


Fig. 3.

The remarkable correspondence between the curves of Lots I and II and particularly between the averages in Fig. 4 demonstrates in a striking manner the part played by inanition as a consequence of vitamin B deficiency.

#### *Attitude of the pigeons.*

The birds of Lot I, at first very active, soon became apathetic, and spent most of the time sitting motionless on the perches with eyes shut and feathers ruffled. The controls behaved quite similarly, which suggests that these symptoms are most probably due to starvation. In the later stages a period of rest-

lessness was observed and the pigeons, apparently searching for a satisfying food, scattered a great part of the diet without, however, eating appreciably. Later, as already mentioned, the food intake sometimes increased, and there was less scattering. Nervous symptoms generally followed immediately afterwards.

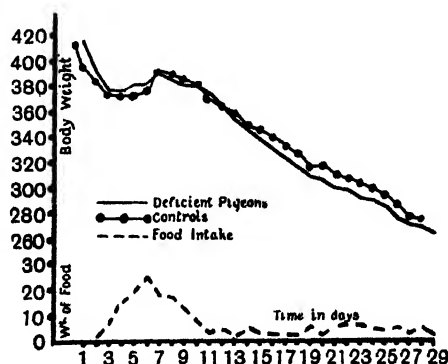


Fig. 4. Average weight and food intake of deficient and control pigeons.

#### *Faeces.*

Changes of consistence and colour of faeces of pigeons on vitamin B-deficient diets have often been described [McCarrison, 1921; Funk, 1921-25; Collazo and Funk, 1924]; the last-named authors have even devised a rough method of determining the vitamin B content of a diet based on the presence or absence of the bright green colour of the faeces; it is said that the green colour disappears in the course of 24 hours after administration of the vitamin. This finding could not be confirmed by the present work, as far as a direct relationship between vitamin B and the consistency and colour of the faeces is concerned. Emerald green coloured liquid faeces were observed as frequently in Lot II as in Lot I and their appearance seemed obviously closely related to insufficient food intake, bacterial infection playing also possibly a rôle, as McCarrison has suggested. On the other hand, faeces of absolutely normal colour and consistency have always been passed by pigeons of Lot I, even in the last stage of the experiment, following the voluntary ingestion of larger quantities of the deficient diet. Green faeces were very rare in the forcibly fed group, appearing only after rejection of food by vomiting. The presence of large amounts of undigested starch in the faeces has been described in vitamin B deficiency by McCarrison [1921] and others. However, Braddon and Cooper [1914] point out that chickens fed one-tenth of their weight of polished rice and developing acute symptoms in the course of 10 days digested 93-98 % of the starch. Similar observations were reported by Eijkman and Hoogenhuyze [1916]. Randoin and Simonnet [1924, 2] consider that pigeons deprived of vitamin B are unable to digest starch. They find, however, also large quantities of starch—more than 60 % of dry weight—in the faeces of

normal pigeons fed on a complete artificial diet. This will be discussed more fully later, and only the results of the analysis of faeces of four lots of pigeons will be given here. The faeces after drying to constant weight were hydrolysed with hydrochloric acid for 5 hours, and the reducing substances determined by Bertrand's method and expressed as "starch" by multiplying the result by the factor 0.9. The faeces were collected between the third and fourth week of the experiment.

Table II.

Pigeons on deficient diets		Pigeons on complete diets	
Lot	% of starch	Lot	% of starch
Lot I (mixed sample)	18.5	Lot II (mixed sample)	18.1
Lot III* (one pigeon)	17.0	Lot IV (mixed sample)	19.6
Lot I† (two pigeons)	20.9	—	—
Average	18.8		18.8

\* It was difficult to collect samples of faeces from this group owing to contamination with vomitus.

† Taken in the last stage of the experiment.

No difference in the digestion of starch can be traced from these figures, which are actually much lower than those recorded by Randoin and Simonnet [1924, 1]. It must be remembered that agar-agar yields reducing sugars on acid hydrolysis.

#### *Nervous symptoms.*

Five out of eight pigeons of Lot I developed acute symptoms: Nos. I *a*, I *b*, I *d*, I *e* and I *h* in the course of 26, 32, 25, 25, and 30 days respectively. Pigeon I *b* had emprosthotonos, the remaining four typical opisthotonos associated with convulsions and "cartwheel" turning. I *a* and I *h* had leg weakness on the day previous to the outbreak of the acute symptoms, in the others the onset was more sudden. Of the three remaining pigeons, one, No. I *c*, received on the 33rd day of the experiment an injection of three units of insulin and was found dead on the following morning. I *f* and I *g* were killed on the 36th day of the experiment for histological examination, without having developed up to that time any acute symptoms. Pigeon I *h* was killed during the convulsions.

#### *Spontaneous cures.*

Pigeons I *a*, I *b*, I *d* and I *e* recovered without being treated, after unmistakable symptoms lasting several hours. Spontaneous cures have already been described by Theiler, Green and Viljoen [1915] and quite recently by Kartascheffsky [1926]. However, as such observations are scarce it seems worth while to give here a more detailed description of an interesting phenomenon. It must be emphasised that the temperature of the animal room being more or less constant at 65–68° F. the possibility of "heat cures" [Peters, 1924] is excluded. Nor was there any probability of a contamination of the diet with a vitamin B-containing substance as the symptoms cleared up at



a time when no food was being consumed by the pigeons. Pigeon I *a* looked quite normal on the day following the convulsions, when an X-ray picture of its gastro-intestinal tract was made. For that purpose a barium meal consisting of 5 g. BaSO<sub>4</sub>, 5 g. of the synthetic diet and 10 cc. of water was administered to the pigeon. It must be pointed out that the barium meal was given after the spontaneous disappearance of the acute symptoms. The pigeon was then quite normal during 5 days and ingested voluntarily during that period 26 g. of the diet. On the sixth day it developed severe acute symptoms—opisthotonos and convulsions associated with impaired respiration—when the bird was killed. The control pigeon II *a* was killed on the following day. Pigeon I *b* developed convulsions and emprostotonos on the 32nd day of the experiment; it was better on the following day; the symptoms reappeared, however, when the bird was taken out of the cage for weighing. Two days later the pigeon was found in convulsions during the morning inspection of the cages and was killed. The control was killed on the following day. No food had been taken during that period by pigeon I *b*. Pigeon I *d* developed acute symptoms on the 25th day; the pigeon was decidedly better on the following day, but head retraction and a fit of convulsions were provoked by mild disturbances of equilibrium. An X-ray picture was taken the next day and a fit of convulsions was also observed during the process. The following three days were uneventful, but the pigeon died suddenly on the 4th night, no food having been ingested after the first onset of convulsions. The control pigeon died a few hours earlier. Pigeon I *e* developed very severe symptoms on the 25th day of the experiment, “cartwheel” turning and convulsions lasting 7 hours with short intervals. The condition improved in the evening and the pigeon looked much better on the following day. An X-ray picture was taken on the next day. The following 7 days were uneventful. On the 5th day after the first attack the pigeon began to eat (no food having been taken for 6 days up to that time) and ingested 16, 13, 7, 12 and 10 g. daily respectively. On the 10th day after the first attack it developed acute symptoms and was killed. The control bird died on the 30th day of the experiment. The very interesting question of spontaneous cures is being now more fully investigated on a larger number of pigeons.

#### *Temperature.*

A progressive fall of the body temperature of pigeons on vitamin B-deficient diets has been observed by many investigators [Dutcher, 1918; McCarrison 1921; Farmer and Redenbaugh, 1925; Barlow, 1926, and others], and those findings are fully confirmed by the present work. Sub-normal steadily decreasing temperatures were also noted by McCarrison in starving birds. The same was found true for the control pigeons of Lot II. The decrease of temperature is roughly equal for both lots up to the onset of acute symptoms. It seems clear, however, that the thermal disturbances occurring during the acute nervous symptoms are of a more complicated character. Spontaneous cures

occurring in four pigeons gave a good opportunity for studying them in detail. Pigeon I *a* had a temperature of 102.2° (Fahr.) on the 23rd day of the experiment and some leg weakness could already be observed. The temperature was 100.4° on the 25th day and 99.5° on the 26th when convulsions occurred. The following day, when the symptoms cleared up, the temperature rose to 104°, *no food having been ingested*. In the morning of the day when the second attack occurred the temperature was 102.9°, it was 91° during the convulsions—a fall of 11.9° (Fahr.) in the course of a few hours. The temperature was unaltered during the first attack of pigeon I *b* (104°) and remained constant to the second. Pigeon I *d* had 104.3° on the 23rd day and 103.1° on the 25th during the convulsions. The temperature rose subsequently and attained 105.1° on the 29th day, on the 30th day it was only 102.5° and the pigeon died in the night (second attack?). Pigeon I *e* had a temperature of 101.4° before the first attack and 99.9° during it, the temperature was 103.2° on the following day, and 104.2° on the 32nd day and then began to drop in spite of a liberal food intake, falling to 101.8° on the 34th day and to 93.7° on the 35th, when the second attack occurred. It is obvious that the control of body temperature is greatly disturbed during the phase with which acute symptoms are associated. A comparison between both lots of pigeons is therefore scarcely justified, but a few average values have been calculated, the temperatures of pigeons having acute symptoms not being included. The data are presented in Table III.

Table III.

Day of Exp.	23	24	25	26	27	28	29	30	31	32	33	34	35
Lot I	103.5°	—	104.6°	104.2°	104.0°	104.3°	104.0°	103.2°	104.2°	104.0°	104.0°	104.3°	104.2° Fahr.
Lot II	—	105.2°	—	105.6°	104.7°	—	105.1°	105.0°	105.8°	105.2°	104.9°	105.0°	105.0° Fahr.

The average temperature is slightly higher in Lot II, but no stress can be laid upon this difference in view of the normal variations that may be encountered. Nevertheless, it seems fair to assume that the thermal changes occurring in pigeons during vitamin B deficiency are twofold; firstly, a progressive decrease which is most probably due to insufficient food intake, and secondly, a profound and rapid fall manifesting itself in the course of a few hours and associated with the onset and gravity of acute nervous symptoms. There seems a possibility that this phenomenon is more directly related to vitamin B. It will be remembered that Farmer and Redenbaugh [1925] observed during convulsions temperatures as low as 92.0° rising to 104° in the course of 6 hours after administration of 0.5 g. of yeast concentrate only. A temperature in the neighbourhood of 103–104° is probably normal for the state of partial starvation occurring in vitamin B-deficient pigeons or in similarly fed controls.

*Examination of the alimentary tract by X-rays.*

The important work of Cowgill and his colleagues [1921; 1923, 1, 2; 1925, 1926] demonstrates clearly that gastric atony is a characteristic symptom in dogs maintained on vitamin B-deficient diets. "Marked retardation and stasis" is reported from cases of human beriberi by Japanese clinicians [Ohomori *et al.*, 1922]. A study of the deficient pigeon in that respect seemed therefore important, and observations of the rapidity of the passage of a barium meal through the alimentary canal of the deficient birds and the controls were made. This was done on the 27th day of the experiment, almost simultaneously with the onset of acute symptoms in five of the pigeons of Lot I. A barium meal consisting of 5 g.  $\text{BaSO}_4$ , 5 g. of the deficient diet and 10 g. of water was placed in the crop by means of a syringe.

An X-ray picture was then taken 1 hour 35 minutes after the meal; that period of time having been determined as the most suitable by a number of observations on normal birds. The control pigeons were treated similarly on the following day. The results were striking in that no appreciable deviation from the normal could be seen in the deficiently fed pigeons. It is impossible to reproduce here all the photographs, but a few typical ones are presented in Plates III and IV. The picture of pigeon Id ((4), Plate IV), deserves special attention, because acute symptoms (convulsions and opisthotonus) were marked whilst the photographs were being taken, so that it was necessary to hold the strongly retracted head in position in order to secure a satisfactory picture. Nevertheless, the food passed through the alimentary canal with normal rapidity, and the strong action of the gut, marked in the photograph, could be most readily followed on the screen. Faeces containing  $\text{BaSO}_4$  were passed at the same time as by the control pigeons. Many photographs of normal pigeons were also taken but no appreciable differences between them and those of deficient birds or controls could be detected. Unfortunately the control II d behaved abnormally, in that no food passed in the gut after 1 hour 35 minutes. This bird died 2 days after the X-ray picture and was most probably ill when the picture was taken.

These results clearly seem to indicate that anorexia in the voluntarily feeding vitamin B-deficient pigeon is not necessarily connected with or accompanied by impaired motile function of the alimentary canal. The question of hunger contractions of the pigeon's stomach is naturally left untouched by the X-ray picture method of investigation. It will be seen later that these results are different from those obtained when the pigeons were fed forcibly on a B-deficient diet.

*Histological examination of nervous system.*

Pathological changes of the nerves in pigeons fed on vitamin B-deficient diets have been described by very many investigators. It was, however, very soon pointed out by Schnyder [1914] that the rapid disappearance of the characteristic symptoms under the influence of vitamin administration can



Pigeon *a*, Lot I, 1 hr. 35 mins. after  $\text{BaSO}_4$  meal. Pigeon *a*, Lot II, 1 hr. 35 mins. after  $\text{BaSO}_4$  meal.



Pigeon *b*, Lot I, 1 hr. 35 mins. after  $\text{BaSO}_4$  meal. Pigeon *b*, Lot II, 1 hr. 35 mins. after  $\text{BaSO}_4$  meal.





(1)

Pigeon *a*, Lot III, 1 hr. 35 mins. after  $\text{BaSO}_4$  meal.



(2)

Pigeon *a*, Lot II, 1 hr. 35 mins. after  $\text{BaSO}_4$  meal.  
Given 1 g. yeast extract daily for 6 days  
after showing acute symptoms.



(3)

Pigeon *a*, Lot IV, 1 hr. 35 mins. after  $\text{BaSO}_4$  meal.



(4)

Pigeon *d*, Lot I, 1 hr. 35 mins. after  $\text{BaSO}_4$  meal.  
X-ray picture taken during convulsions.



scarcely be due to the repair of degenerated nervous tissue. A similar view is clearly expressed by McCarrison [1921]. Histological examination of pigeons I *f*, II *f*, II *g* and III *d* was kindly undertaken by Dr Woollard in the Department of Anatomy, University College, who reports as follows:

"An histological examination was made of a pigeon that had been on a deficient diet (*f*, Lot I) for 35 days. The bird had exhibited no nervous phenomena during life and an examination of the intermuscular nerves and nerve endings showed an abundance of normal nerves and endings. In fact no deviations from the normal could be recorded in this case. Two control pigeons (*g* and *f*, Lot II) which had received the same amount of food as the birds on the deficient diet voluntarily consumed were also examined. These birds had also been given yeast extract. The examination of the first bird of this group showed perfectly normal intermuscular nerves and nerve endings. The bird was fairly well nourished. The second bird of this series was extremely wasted, and in this case there could be observed considerable changes in the terminal nerves and endings in the muscles. In all cases the tissue examined came from the muscles of the leg. A pigeon from Lot III (*d*) was also examined, it having exhibited typical head retraction for some days. This forced movement can easily be overcome and the head made to assume a normal position. However, the slightest stimulus brings about a reproduction of the retraction. When the bird is held head downwards, the head under the influence of gravity will gradually sink, but again the slightest stimulus will restore the retraction. Such a position involves alteration of the tone in various muscle groups. There is no paralysis and the position of the head bears most resemblance to the forced movements that can be elicited from the labyrinth, from the mid-brain, and from the cortex. Indeed there is little neurological ground for supposing that head retraction depends on changes in the nerves of the neck muscles. The examination of intermuscular nerves in this case was unfortunately not entirely satisfactory, but the preparations were sufficient to show large numbers of normal fibres and endings. To what extent there were degenerative changes it is not possible to say from the specimens. Of the series studied it is to be remarked that the specimen which showed the most advanced changes was the bird that had received vitamin *plus* a quantity of food equal to the voluntary intake of the birds on the deficient diet."

It seems evident from this study that starvation and the wasting associated with it are largely if not entirely responsible for the pathological nerve changes observed in vitamin B deficiency in pigeons. This conclusion provides further support for the views recently put forward by Woollard [1927]. It cannot be sufficiently emphasised that no nerve regeneration is possible in the course of a few hours, and that the nervous symptoms, vanishing so rapidly under the action of vitamin B or even disappearing spontaneously must be due to a quite different cause. There is no logical impediment, however, in assuming that the paralytic form of pigeon's beriberi which is not curable or very hardly curable by vitamin administration may be associated with nerve degeneration.



*Blood-sugar.*

Funk and Schönborn [1914], Funk [1920], Randoin and Lelesz [1925], Collazo [1923], and others observed hyperglycaemia in pigeons on vitamin B-deficient diets. Funk [1914] considers vitamin B as specially connected with the carbohydrate metabolism. Control determinations on partially starving pigeons were done by Collazo [1923], who found in such pigeons much the same increase of blood-sugar as in pigeons on vitamin B-free diet. He also cites several papers dealing with starvation-hyperglycaemia.

The blood-sugar was estimated in four pigeons of Lot I and in four pigeons of Lot II by the reliable method of Hagedorn and Jensen and the following figures were obtained.

Table IV.

Lot I		Lot II	
Pigeon No.	Blood-sugar	Pigeon No.	Blood-sugar
I e*	0.214 %	II b	0.251 %
I f	0.222 %	II f	0.224 %
I g	0.222 %	II g	0.248 %
I h*	0.228 %	II h	0.186 %
Average	0.221 %	Average	0.227 %

\* Blood taken during convulsions.

There is no difference between the two lots of pigeons. The blood-sugar content of the normal pigeon is 0.21 % according to Collazo [1923], and 0.185 % according to Honeywell [1922]. The slight increase observed in Lots I and II is most probably due to inanition.

*Adrenaline content and weight of adrenals.*

A hypertrophy of the adrenal glands in vitamin B-deficient pigeons was first observed by McCarrison [1921] and his observation has been confirmed by many investigators. McCarrison found also an increased output of adrenaline in vitamin B deficiency and considers it as playing a part in the production of oedema in cases of "wet" avian and human beriberi, whilst Funk [1924] ascribes to it a rôle in the causation of hyperglycaemia. It will be remembered that hypertrophy of the adrenals was also repeatedly observed in cases of inanition [Vincent and Hollenberg, 1920; McCarrison, 1921 and Findlay, 1921], but, on the other hand, pure starvation is always associated with vitamin B deficiency. Beznak [1923], while confirming the hypertrophy of the adrenals in beriberi pigeons, could find no increase in the size of adrenals in starving pigeons receiving only vitamin B (in the form of yeast). He therefore considers that hypertrophy of the adrenals is definitely caused by vitamin B deficiency, but points out, since he could find no increase in the adrenaline content, that probably the cortical tissue alone hypertrophies.

The results of a few determinations made on pigeons from Lots I and II (two determinations in each lot) are given in Table IV. The method of Baker and Marrian [1927] was used.

Table V.

	No. of pigeon	Weight at beginning g.	Weight at end g.	Weight of adrenals g.	Adrenaline $\times 10^{-2}$ mg.	Adrenaline in mg./g. gland
Lot I	I h	383	233	0.0691	17.8	2.57
	I e	457	281	0.0532	11.85	2.23
Lot II	II h	355	220	0.0395	17.6	4.45
	II b	443	298	0.0352	11.4	3.24

As far as these figures go they tend to confirm the observations of Beznak, but they are obviously inadequate and further work seems desirable before the conclusion is adopted that the hypertrophy of the adrenals is due specifically to a deficiency of vitamin B.

#### *Blood-calcium.*

A few estimations of blood-calcium were made with the view in mind that the convulsions might possibly be associated with a hypocalcaemia. Actually a slightly increased amount was found present in the blood of the pigeons lacking vitamin B. The blood-calcium was determined by the Clark-Collip [1925] modification of the excellent Kramer-Tisdall [1921] method. The blood was drawn from the wing vein.

Table VI.

No. of pigeon	Blood-calcium mg./100 c.c.	Remarks
Lot I	I a	12.1 Blood taken during convulsion
	I e	11.4
	I f	10.0 On 36th day of "vitamin B"-free diet
	I g	10.0 " " " "
Average: 10.9		
Normal aver. 8.2	Normal	8.2
	"	8.2
Lot II	II a	8.3
	II b	8.4
	II g	8.3
Average: 8.3		

Ungar [1927] reports no appreciable difference between the blood-calcium levels in normal pigeons and others during beriberi convulsions.

#### *Starch-hydrolysing enzymes of the alimentary tract.*

A complete loss of the amylolytic and lipolytic, and a marked decrease of the proteolytic enzymes in pigeons fed on vitamin B-deficient diets has been reported by Farmer and Redenbaugh [1925]. Previously various investigators tried to connect the outstanding features of vitamin B deficiency with changes in the quantity and activity of the digestive juices. That no direct relation exists between vitamin B and the secretory function of the digestive glands has been conclusively demonstrated by the work of Anrep and Drummond [1921] and Cowgill and colleagues [1921 to 1926]. On the other hand, it appeared to us that the almost identical behaviour of the pigeons in our Lots I and II was hardly compatible with the findings of Farmer and Redenbaugh. It seemed, therefore, important to repeat their work. This was done, using three normal pigeons kept previously in a large aviary on poultry mixture diet, four pigeons of the deficient Lot I, one pigeon of the deficient Lot III and four pigeons of the control Lot II. The technique of Farmer and

Redenbaugh was closely followed except that the pancreas and first 6 inches of the intestines were not minced in a meat mincer but ground with sand in a mortar. The glycerol extracts were centrifuged after 4 weeks' maceration and the amylolytic properties determined according to Long and Johnson [1913]. The results are given in Table VII.

Table VII. *Starch digestion.*

Incubation at 37°.					
<i>Normal pigeons.</i>					
Pigeon No.	After 10 mins.	After 45 mins.	After 80 mins.	After 120 mins.	
Normal I	blue	blue	purple-red	red	
„ II	blue	blue	purple	purple-red	
„ III	blue	purple	pale pink	very pale pink	
<i>Deficient pigeons (Lot I and Lot III).</i>					
All killed during convulsions with the exception of III h.					
I a	blue	purple	pale pink	very pale pink	
I b	blue	faint yellow	faint yellow	faint yellow	
I c	purple-blue	faint yellow	faint yellow	faint yellow	
I h	blue	purple-red	faint yellow	faint yellow	
III h	blue	purple-red	faint yellow	faint yellow	
<i>Controls (Lot II).</i>					
II a	blue	blue	reddish purple	red	
II b	blue	purple	pale purple-red	faint yellow	
II d	blue	faint yellow	faint yellow	faint yellow	
II h	blue	purple-blue	pink	very pale pink	

Our observations are entirely different from those made by Farmer and Redenbaugh, but are in agreement with other findings reported in the present paper, all of which demonstrate clearly that the digestion, at any rate of starch, is normal in a vitamin B-deficient pigeon. The extracts of the organs of the normal pigeons are, if anything, rather less active than those of the deficient birds or of the controls. This somewhat unexpected result is probably explained when one takes into consideration the great wasting which the deficient birds have suffered, so that the extracts are simply more concentrated, as the wasting involves chiefly inactive material such as the fat of the pancreas and muscular wall of the gut. It must be remembered that in the experiment of Farmer and Redenbaugh the birds were fed forcibly and that they were eating voluntarily in the present experiment. A highly active extract was, however, obtained also in the case of pigeon III h belonging to a group that had been forcibly fed. It is not easy to account for the results of Farmer and Redenbaugh, but what is believed to be an inconsistency in their results may here be pointed out. It seems very difficult to understand how pigeons having, according to their opinion, completely lost the power of utilising starch and fats and to a marked extent that of splitting proteins could be kept for an average period of 27 days on a vitamin B-free diet of autoclaved grain with an average loss of only 3.7 g. daily. The average daily food intake was 25 g. Even leaving out of consideration the alleged partial impairment of the utilisation of proteins and total of fats, and only deducting from that figure 70 % for non-metabolised

starch it is easy to calculate that only 7.5 g. of the diet containing a considerable amount of roughage were in reality available for the pigeons. That quantity was furthermore greatly diminished by the fact that pigeons "frequently regurgitated a considerable amount" and was surely not sufficient to spare daily 5.6 g. of body tissue. A similar recalculation in the case of the lard diet fed in quantities of 15 g. daily and containing 39 % of starch and 21 % of lard would lead, if the assumptions of Farmer and Redenbaugh were correct, to an even more striking result as the daily loss in that group of pigeons was only 1.17 g. (McCarrison's figures for starvation are 9.3 g.). It will be remembered that the control pigeons of Lot II receiving a liberal supply of vitamin B in the form of marmite and with an average daily food consumption of 6.1 g., none of which was regurgitated, presented an average daily loss of 5 g. of weight. There is also another statement in the paper of Farmer and Redenbaugh with which we cannot agree, namely, that "the gastro-intestinal tract of the starved pigeon is normal and empty with little or no permanently decreased efficiency of the digestive enzymes. In the pigeons with polyneuritis ... the gut usually shows degenerative changes." That the gastro-intestinal tract undergoes very marked changes in starvation and can by no means be considered as normal has been established beyond doubt by the researches of McCarrison on a great number of pigeons.

#### LOTS III AND IV.

Pigeons fed forcibly with a vitamin B-deficient diet almost invariably vomit a great deal of it, so that accurate estimates of food intake are difficult to make. Similar rejection of food is sometimes seen in birds feeding voluntarily on the deficient diet, but was not observed in our Lot I. Such experiments are therefore much more liable to error than those carried out when the ingestion is voluntary. It is also, in our opinion, desirable that the abnormal method of feeding, particularly when applied to an animal that exhibits a distaste for the particular food, and its possible effects on the animal should be always borne in mind when the results of such experiments are being discussed. This applies to the results obtained in the present work with Lots III and IV so that they will be only briefly treated. Two interesting features must, however, receive attention. The two lots of pigeons were fed daily 20 g. of the finely ground diet mixed with a sufficient amount of water to permit passage through the syringe and rubber tube. Approximately 30 cc. of water were used. Pigeons of Lot IV were also given daily 1 g. of marmite. The pigeons of the deficient group began to regurgitate the food on the 5th to 9th day of the experiment. The vomiting was purely voluntary and was effected in the same manner as when feeding the young. Some birds vomited immediately after being fed, some only after a few hours had elapsed. Vomiting occurred also to a certain extent in the control Lot IV and was most probably due here to the discomfort caused by the large bulk of food being introduced at once in the crop. It must be emphasised here that vomiting never occurred in the

control Lot II—the quantities of food administered being much smaller than in the case of Lot IV. A marked retention of the food in the crop was always observed in the deficient pigeons in the later stages of the experiment and especially just prior to the onset of the acute symptoms when they were unable to execute the movements of the head and wings necessary to expel the contents of the crop.

### *Weight.*

The initial average weights and the average weights on the 18th day of the experiment of both lots are given in Table VIII. After that date food retention associated with acute symptoms was occurring frequently and the weight figures were therefore unreliable.

Table VIII.

	Initial weight	Weight on the 18th day	Average daily loss
	g.	g.	g.
Lot III*	417	330	5.1
Lot IV	416	388	1.6

\* Average of six pigeons—III *e* died on the 13th day of the experiment and III *c* developed acute symptoms and was given marmite on the 15th day.

The loss in weight of the pigeons of Lot IV was due to the occurrence of vomiting and not to an insufficient food intake, as gain in weight was observed in those pigeons which did not vomit.

It is very interesting to note that the average daily loss of Lot III is only slightly lower than that of Lot I—forcibly fed pigeons practically behave as if they were feeding voluntarily in the respect that only a certain definite amount of the food deposited in the crop is allowed to pass further down the alimentary canal and the remainder is expelled.

### *X-ray picture.*

X-ray pictures of the gastro-intestinal tract of pigeons III *a*, III *b*, III *c* and their controls, and III *f*, III *g*, III *h* and their controls, were made under conditions quite similar to those already described on the 12th and 15th days of the experiment respectively. A marked difference between the deficient lot and the controls could be observed in all the cases. It is illustrated in two typical photographs of pigeons III *a* and IV *a* reproduced here ((1) and (3), Plate IV). 1 hour and 35 minutes after the meal the stomach alone is faintly visible in the deficient pigeon and no food is seen descending the oesophagus: in the control pigeon the oesophagus can be easily detected, the stomach and intestines are full and the barium meal is approaching the rectum. Pigeon III *a* was then given daily marmite, beginning with the 18th day of the experiment, and an X-ray photograph was taken on the 24th day showing normal passage of the food through the alimentary tract ((2), Plate IV). The retardation of the passage of food in the pigeons that have been forcibly fed might be due to an impairment of the mechanism for emptying the crop in a normal manner or

to a voluntary inhibition of this process. It might also be due to an absence of the stomach movements, and to decreased peristalsis of the intestines. We are inclined to favour the first view, for the reason that any food passing to the intestines was normally digested, and normal peristaltic movements could be seen on the screen when after a sufficient period of time the Ba meal was present in the intestines. If the crop was functioning normally a passive filling of the empty intestine a short time after administration of the Ba meal could be expected. It is believed that the impaired functioning of the crop is not due to muscle atrophy or nerve degeneration, because the condition improves rapidly as was observed by many investigators after administration of vitamin B in a suitable form.

#### *Acute symptoms.*

The acute symptoms occurred sooner in Lot III than in Lot I. Four pigeons, III *c*, III *d*, III *f*, III *g*, presented the typical picture after 15, 37, 19 and 22 days respectively with an average of 23 days as compared with 28 days in the case of those voluntarily feeding. Two other pigeons, III *a* and III *b*, developed paralysis and leg weakness, but no symptoms of head retraction after 17 days and 22 days. III *a* quickly recovered after administration of marmite, but III *b* died on the following day. Pigeon III *c* died from other reasons on the 13th day of the experiment. Pigeon III *h* was killed on the 26th day without showing acute symptoms.

#### DISCUSSION.

Reviewing the results of this investigation as a whole it seems apparent to us that many of the characteristic symptoms exhibited by pigeons fed on diets deficient in vitamin B are direct consequences of the failure to consume sufficient food to maintain the body weight. In this respect these experimental results are confirmatory of those reported by Drummond and Marrian [1926] derived from somewhat similar studies of the rat. In that paper evidence was presented to disprove a number of theories ascribing to vitamin B an essential influence on the production or functioning of the oxidative mechanisms of the tissues. It is believed that the present paper serves to throw doubt on another group of theories, namely, those which relate vitamin B to the digestive and assimilatory functions of the organism. In the first place, the view expressed by various authors that vitamin B is necessary for the proper utilisation of carbohydrates is unsatisfactory in that it ignores the fact that more than 50 % by weight of the molecule of proteins is metabolised along the path of carbohydrate breakdown. Such a view implies that the mechanism responsible for the utilisation of carbohydrates arising from proteins remains normal in vitamin B deficiency, whereas a distinct and independent mechanism involved in the metabolism of exogenous carbohydrates undergoes a profound disturbance. It is very difficult on that basis to account satisfactorily for the results of Randoin and Simonnet [1924, 1, 2], who reported that they were able to keep pigeons in perfect health for a period of 3½ months on a diet deficient in vitamin

B and in carbohydrates but containing 54 % of protein and 32 % of fat, or on a diet almost identical to that used in the present work<sup>1</sup>, but fed in amounts of 75 g. per pigeon daily, from which the pigeons were able to "pick out" the protein and other constituents, leaving the carbohydrate part untouched. Why a reduction of the daily intake of that diet to 20 g. should hasten the onset of acute symptoms—which then appeared after 2 months—remains obscure. A similar difficulty is encountered when discussing the work of Funk and his colleagues [Funk, 1914; Funk and Paton, 1922; Collazo and Funk, 1924] on the subject. In a recent paper Funk and Collazo [1925] report that pigeons receiving a constant amount of vitamin B and kept in weight equilibrium on a diet containing 12.5 g. protein showed an increase in body weight roughly proportional to the amount of protein ingested when offered dietaries containing 25, 50 and 75 % of protein, the other variable component being starch. It seems clear that in these cases the food values of the diets and their influence on the weight curve were not properly controlled. A review of our experimental observations makes it evident that no results corroborating these theories have been obtained. The actual food intake of a pigeon from which vitamin B is withheld, be it forcibly fed or partaking freely of the diet, is greatly diminished, but the ingested quantum is metabolised just as completely as in a pigeon receiving liberal amounts of vitamin B in the form of yeast extract; and this is true even in the stage of the deficiency disease where the characteristic convulsions appear. The similarity of the curves for food intake and body weight, the similar rates of passage of a meal through the intestines, the blood-sugar levels and composition of the faeces all support this view. Furthermore, it is believed that the diminished food intake and subsequent partial starvation are sufficient to explain any hyperglycaemia encountered in vitamin B-deficient pigeons. This confirms the findings of Collazo [1923], but the views expressed by Funk and Schönborn [1914], Funk [1920], Randoin and Lelesz [1925] and others cannot be shared. In fact, the starvation factor was not taken into account by these investigators. Passing now to the relation of vitamin B deficiency to the function of the digestive glands it is obvious that a normal digestion of starch observed in beriberi pigeons suggests the existence of a normally functioning digestive apparatus. This view is supported by the demonstration of the presence of a highly active amylolytic enzyme in the pancreas and intestines of beriberi pigeons, confirming the finding of Tiger and Simonnet [1921] and Rothlin [1922]. That the gastric secretion has a normal composition throughout the course of vitamin B avitaminosis has been conclusively demonstrated by Bickel [1922]. Anrep and Drummond [1921] and Cowgill and Mendel [1921] presented experimental data leading them to deny any stimulating influence of vitamin B on the digestive glands. It seems that the evidence weighs against the opinion and findings of Farmer and Redenbaugh [1925].

<sup>1</sup> Randoin and Simonnet used potato starch instead of rice starch and reach the curious conclusion that a vitamin B-deficient pigeon can digest the latter but not the former.

We have been greatly disturbed during this work by the number of spontaneous cures of the typical beriberi convulsions that have occurred. In our opinion such cures are much more frequently encountered in pigeons fed on an artificial diet such as we have employed than in those fed in the more customary manner on polished rice.

That a substance indistinguishable in all its characteristics from what we are accustomed to call vitamin B is present in the organism under normal conditions seems certain. Even after a long period of vitamin B deficiency the body stores are not completely exhausted as was shown by the interesting work of Funk [1914], since confirmed by various authorities [Theiler, Green and Viljoen, 1915; Hess and Takahashi, 1921; Abderhalden, 1922]. It will be remembered that Funk extracted the tissues of pigeons after death in beriberi convulsions in a manner similar to that used for extracting rice polishings and obtained extracts which had marked curative effects when administered to other pigeons in convulsions. That result recalls the observation of Baker, Dickens and Dodds [1924], that large quantities of insulin can be extracted from the organs of patients dying in diabetic coma.

There is a possibility that there may be a liberation of the reserves of vitamin B in the tissues during periods when there is none in the diet and when there is considerable tissue wasting; that such liberation may occur is suggested by the fact that pigeons and fowls do not exhibit in pure starvation (which is also obviously a vitamin B deficiency) the characteristic nervous symptoms of vitamin B deficiency unless they are "washed out" by administration of large quantities of water [Eijkman and Hoogenhuyze, 1916; Chamberlain, Bloomergh and Kilbourne, 1911; Chamberlain and Vedder, 1911]. The question immediately arises whether what is perhaps the most characteristic symptom of vitamin B deficiency, namely, the loss of the desire to partake of the deficient food, is to be looked upon merely as a morbid phenomenon or as an attempt of the organism to liberate by starvation and subsequent tissue wasting the body stores of the lacking vitamin bound in the tissues so as to be inaccessible to the animal under normal conditions and thus make available a substance essential for life. The occurrence of spontaneous cures on diets such as we employed suggests that there may be a liberation following a convulsive seizure which gives temporary relief and sometimes leads to a transient return of appetite. Further investigation must follow before an opinion on this question can be formed. It seems evident that almost all the pathological changes observed in beriberi pigeons are directly attributable to inanition. There are, however, certain differences between a vitamin B-deficient pigeon and one partially starving but receiving a liberal supply of the vitamin, namely, the occurrence in the former of two well-defined symptoms which in the order of their appearance are: (1) a diminished desire to partake of *the deficient food*, (2) symptoms known as opisthotonos, emprosthotonos, convulsive seizures, "cartwheel" turning. Leg weakness is also frequently present, but is not characteristic as a symptom. It yields only with difficulty to vitamin B treatment and is often observed in the premortal stage in control birds.



Regarding the fluctuations of temperature that are associated with the convulsion phase of vitamin B deficiency which seem of different origin from the fall that is a consequence of inanition, and the hypertrophy of the adrenals, we are not yet prepared to express an opinion. Much evidence is against associating the nervous symptoms described under (2) with the nerve lesions considered by many authorities as a characteristic consequence of vitamin B deficiency. Identical lesions have been found by Dr Woollard on histological examination of our control pigeons on restricted food but receiving a liberal supply of yeast extract, whereas they are often lacking in beriberi pigeons (McCarrison). Finally, the prompt recovery following administration of vitamin B excludes any possibility of nerve regeneration and strongly suggests that the mechanism responsible for the appearance of the symptoms or for their retrogression is of a different character.

It may be stated with some certainty that no existing theory is adequate satisfactorily to explain the origin of the nervous syndrome characteristic of vitamin B deficiency or the rôle played by vitamin B in the normal organism. As yet we wish to make no contribution to the theoretical side; we would, however, support the opinion of Karr [1920] that "some relationship exists between the desire to partake of food and the amount of the so-called water-soluble vitamin ingested."

#### SUMMARY.

(1) A method of studying vitamin B deficiency is described in which the effects of inanition are checked by a suitable control. The method, as applied to pigeons, consists in determining their voluntary food intake when kept on a vitamin B-deficient but otherwise complete synthetic diet and administering artificially the same amount of food to individual control birds of the same weight which receive in addition a liberal supply of vitamin B in concentrated form.

(2) As a result of such studies the decrease in body weight accompanying vitamin B deficiency in pigeons is considered as solely attributable to partial starvation.

(3) No difference could be observed in the utilisation of food between the deficient birds and the controls.

(4) Green faeces are considered to be due to insufficient food intake and possibly bacterial infection.

(5) The "ruffled feathers" attitude of the deficient birds is due to inanition.

(6) No difference was found between the blood-sugar levels of the two groups. The hyperglycaemia, if any, is due to inanition.

(7) A hypertrophy of the adrenals in vitamin B deficiency is considered as probable, but no difference in the adrenaline content is found.

(8) No difference could be observed in the rate of passage of a barium meal through the gastro-intestinal tract of birds feeding on the deficient diet voluntarily and of the controls receiving the vitamin.

(9) A marked slowing of the rate of passage of the barium observed in the case of artificially fed birds is attributed not to intestinal stasis but to a "barrier action" of the crop.

(10) The gradual fall of the body temperature observed in deficient pigeons is due to partial starvation.

(11) A loss of temperature control during the convulsive stage of beriberi is considered as probable.

(12) Highly active amylopsin preparations are obtained from the pancreas and intestines of deficient birds killed during convulsive seizures, as well as from controls.

(13) The view that vitamin B controls the carbohydrate metabolism is not corroborated.

(14) Lack of appetite for *the deficient food* and acute nervous symptoms are characteristic of vitamin B deficiency in the pigeon.

(15) No relation could be traced between the occurrence of acute nervous symptoms and degenerative changes in the nerves. Such changes were present in both groups of pigeons and are attributable to inanition.

(16) No existing theory is considered as giving a satisfactory explanation of the rôle of vitamin B in the normal organism, or of the true nature of the conditions induced by its deficiency.

One of us (S. K. K.) desires to thank the International Health Board of the Rockefeller Foundation for a Fellowship during the tenure of which the work here reported was carried out. The authors wish to thank the Medical Research Council for a grant from which the expenses were defrayed. They are also indebted to Miss K. W. Wilcox, M.Sc., for help during part of this work, and to Mr Melville for the X-ray examinations.

#### REFERENCES.

- Abderhalden (1922). *Arch. ges. Physiol.* **195**, 432.  
Anrep and Drummond (1921). *J. Physiol.* **54**, 349.  
Baker, Dickens and Dodds (1924). *Brit. J. Exp. Path.* **5**, 327.  
Baker and Marrian (1927). (*Unpublished results.*)  
Barlow (1926). *Amer. J. Physiol.* **78**, 322.  
Beznak (1923). *Biochem. Z.* **141**, 1.  
Bickel (1922). *Klin. Woch.* **1**, 110.  
Braddon and Cooper (1914). *J. Hyg.* **14**, 331.  
Chamberlain, Bloombergh and Kilbourne (1911). *Philippine J. Sci.* **6 B**, 177.  
Chamberlain and Vedder (1911). *Philippine J. Sci.* **6 B**, 251, 395.  
Clark and Collip (1925). *J. Biol. Chem.* **63**, 461.  
Collazo (1923). *Biochem. Z.* **134**, 194.  
Collazo and Funk (1924). *Med. Doswiadczenia i spol.* **3**, 359; *J. Metab. Res.* **5**, 187, 195.  
Cowgill (1921). *Amer. J. Physiol.* **57**, 420.  
—— (1923, 1). *J. Biol. Chem.* **58**, 725.  
—— (1923, 2). *Amer. J. Physiol.* **66**, 164.  
Cowgill, Duel and Smith (1925). *Amer. J. Physiol.* **73**, 106.  
—— ——— (1926). *Amer. J. Physiol.* **77**, 389.  
Cowgill and Mendel (1921). *Amer. J. Physiol.* **58**, 131.

- Drummond and Marrian (1926). *Biochem. J.* **20**, 1229.
- Dutcher (1918). *J. Biol. Chem.* **36**, 63.
- Eijkman and Hoogenhuyze (1916). *Proc. R. Akad. Wetensch., Amsterdam*, **18**, 1467.
- Farmer and Redenbaugh (1925). *Amer. J. Physiol.* **75**, 27, 45.
- Findlay (1921). *J. Path. Bact.* **24**, 175.
- Funk (1914). *Z. physiol. Chem.* **89**, 373, 378.
- (1920). *J. Physiol.* **53**, 247.
- (1921-25). Article on vitamins in *Handbuch der Bioch. Arbeitsmethoden*, **4**, ix.
- (1924). *Die Vitamine* (Dritte Auflage, Bergmann).
- Funk and Collazo (1925). *Chem. Zell. Gew.* **12**, 195.
- Funk and Paton (1922). *J. Metab. Res.* **1**, 737.
- Funk and Schönborn (1914). *J. Physiol.* **48**, 328.
- Gulick (1922). *Amer. J. Physiol.* **59**, *Proc. Amer. Physiol. Soc.* 483.
- Hess and Takahashi (1921). *Biochem. Z.* **122**, 193.
- Honeywell (1922). *Amer. J. Physiol.* **58**, 152.
- Karr (1920). *J. Biol. Chem.* **44**, 255.
- Kartascheffsky (1926). *Arch. ges. Physiol.* **215**, 197.
- Kramer and Tisdall (1921). *J. Biol. Chem.* **47**, 475.
- Long and Johnson (1913). *J. Amer. Chem. Soc.* **35**, 895.
- McCarrison (1921). *Studies in deficiency disease* (Oxford Med. Publ.).
- McCollum and Davies (1915). *J. Biol. Chem.* **23**, 181.
- Ohomori, Ohhashi, Nakanishi, Hara and Ota (1922). *Japan Med. World*, **2**, 128.
- Peters (1924). *Biochem. J.* **18**, 858.
- Randoin and Lœlesz (1925). *Compt. Rend. Acad. Sci.* **180**, 1366.
- Randoin and Simonnet (1924, 1). *Bull. Soc. Chim. Biol.* **6**, 601.
- (1924, 2). *Compt. Rend. Acad. Sci.* **179**, 700.
- Rothlm (1922). *Z. physiol. Chem.* **121**, 300.
- Schnyder (1914). *Arch. Verdauungskrankh.* **20**, 147.
- Theiler, Green and Viljoen (1915). *3rd and 4th Director Veter. Res. Rep. Dept. Agric., Union S. Africa*, 9.
- Tiger and Simonnet (1921). *Bull. Soc. Chim. Biol.* **3**, 580.
- Ungar (1927). *Biochem. Z.* **180**, 357.
- Vincent and Hollenberg (1920). *Endocrin.* **4**, 408.
- Woollard (1927). *J. Anat.* **61**, 414.

# **LXXXIX. THE PHYSIOLOGICAL RÔLE OF VITAMIN B. PART IV.**

## **THE RELATION OF CERTAIN DIETARY FACTORS IN YEAST TO GROWTH OF RATS ON DIETS RICH IN PROTEINS.**

BY ALI HASSAN AND JACK CECIL DRUMMOND.

*From the Department of Physiology and Biochemistry,  
University College, London.*

*(Received March 23rd, 1927.)*

### **INTRODUCTION.**

DRUMMOND, CROWDEN and HILL [1922] reported that rats and cats showed a subnormal rate of growth when fed on diets composed largely of caseinogen, but containing what were considered to be adequate amounts of the inorganic salts and vitamins. These observations seemed definitely related to those of Hartwell [1921, 1924, 1925] made during an investigation of mammary secretion in rats, in which she traced a quantitative relation between the protein level of the diet and some factor or factors showing a similar distribution in foodstuffs to vitamin B. A somewhat similar conclusion was reached by Reader and Drummond [1926] who obtained satisfactory growth in rats only when the ratio of protein to the yeast extract used in the diet was about 5 or less.

A consideration of these and other results did not lend support to the opinions that have been expressed to the effect that the vitamin requirement stands in relation to the amount of carbohydrate ingested or metabolised, and provisional support was given to Hartwell's view that it is concerned with the fate of the proteins or their constituent units as a whole rather than with the metabolism of the nitrogen-free residue as was at one time suggested. Another suggestion has been advanced by McCollum and Simmonds [1925] who ventured the view that the subnormal rate of growth noted in our experiments was due to an improper balance between the calcium and phosphorus in the diet, leading to "high-phosphorus" rickets. Such a possibility had suggested itself to us, especially as in the first set of experiments some lameness was noted in the experimental cats, but Reader and Drummond were unable to detect any failure of calcification on X-ray examination of the rats reported in the paper published in 1926. Furthermore, it seemed

unlikely that supplementing the protein-rich diet with additional yeast extract itself rich in phosphorus and poor in calcium could improve the balance of these elements.

It is, therefore, not by any means clear what the action of the yeast extract is in these experiments and further tests were made in order to throw some light on the matter.

Some of Hartwell's results point to the yeast factors operating in her experiments being organic in nature. She was unable to reproduce the effect of the yeast extract by adding salts; the failure of calcium being significant in the light of McCollum's suggestions. Nevertheless, it seemed desirable to ascertain whether the phenomenon observed by us had the same origin as that studied by her, and to determine whether the yeast extract served to adjust the salt or acid-base balance of the diet; it must be remembered that the metabolism of large quantities of caseinogen calls for the neutralisation of considerable quantities of sulphuric and phosphoric acids.

Another point to be studied was obviously the nitrogen metabolism of animals failing to grow satisfactorily on a diet inadequately balanced as regards protein and the yeast factor, in order to find out whether there was any failure to digest and absorb proteins or a disturbance of metabolism reflected in changes in the distribution of nitrogen in the urine.

It will be convenient to deal with this part of the investigation first.

#### EXPERIMENTAL.

*Nitrogen balance.* Three young male rats of the same litter were placed in metabolism cages of the Hopkins pattern. Analyses of the excreta were made every few days, decomposition of the urine having been prevented by collection in dilute sulphuric acid and a small quantity of toluene. The

Table I.

	No. Days in period	Total food con- sumption	Average daily con- sumption	Nitrogen- intake	Nitrogen-output		Nitrogen balance	Change in wt. of rat
		Dry wt. g.	Dry wt. g.		Faeces g.	Urine g.		
Control rat on	4	45.85	11.46	1.9890	0.1364	1.2110	+0.6416	+ 5
Diet A (low	4	53.00	13.25	1.9867	0.1073	1.3020	+0.5774	+ 8
protein, low	4	59.99	14.99	1.9378	0.1635	1.3720	+0.4023	+ 7
yeast)	4	68.03	17.01	2.0651	0.1521	1.3685	+0.5445	+10
	5	76.24	15.24	2.4551	0.1786	1.6660	+0.6104	+ 9
Rat on Diet E	4	32.18	8.04	3.3051	0.1258	2.7895	+0.3898	+10
(high protein,	4	34.94	8.73	3.5891	0.1416	3.840	-0.4265	-12
low yeast)	4	41.97	10.49	3.6682	0.1330	3.2340	+0.3012	+ 7
	4	44.59	11.14	3.9796	0.1455	3.4577	+0.3764	+13
	4	43.26	10.81	3.8808	0.1489	3.6855	+0.0464	+ 1
Rat on Diet C	4	36.01	9.00	3.9287	0.1335	3.062	+0.7432	+15
(high protein,	4	39.31	9.82	4.0695	0.1910	3.780	+0.0785	+ 5
high yeast)	5	46.25	9.25	4.4527	0.2119	4.0135	+0.2273	+ 4
	4	46.36	11.59	4.4569	0.1741	4.284	-0.0012	+ 3
	4	47.05	11.76	4.5234	0.1650	3.983	+0.3754	+ 5

faeces were removed daily, dried at 100°, powdered, and a sample weighed out for analysis.

Three diets of the composition used by Reader and Drummond were employed.

	Low protein, low yeast	High protein, high yeast	High protein, low yeast
	(A)	(C)	(E)
Casoinogen	20	70	70
Rice starch	70	20	20
McCullum's salt mixture	5	5	5
Yeast extract	4	16	4
Cod-liver oil	2	2	2

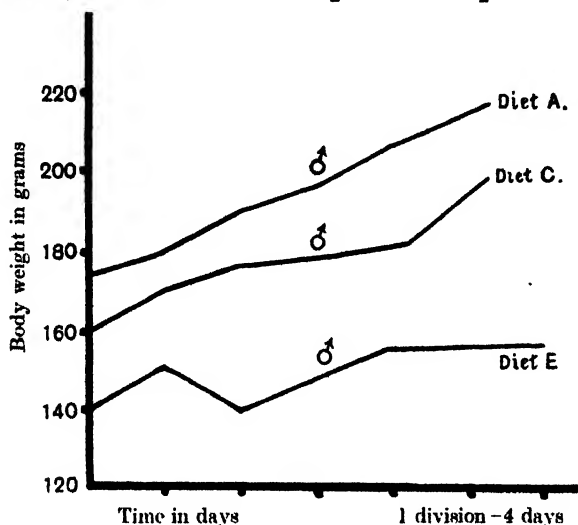


Fig. 1.

The growth of the three animals is shown in Fig. 1, the curves of which are similar in general features to those recorded previously from this laboratory. The analyses and figures in Table I indicate clearly that there is no appreciable disturbance of the digestion and absorption of protein in the case of rats showing unsatisfactory growth on diets rich in protein but with an inadequate amount of the related yeast factor (Diet E). The average faecal excretion of nitrogen daily in each group is—Diet A, 0.0351; Diet C, 0.0417; Diet E, 0.0297 g.; figures well within the normal range for animals of this weight.

*Distribution of nitrogen in the urine.* In this experiment groups of several animals were placed in metabolism cages in order to obtain sufficient urine for a full examination. Four groups were employed, three of which were fed on the diets already described whilst a fourth was given a modified diet containing a normal proportion of protein (20 parts) but the larger amount (16 parts) of yeast extract. This was necessary in order to control the effect of the extra yeast with its high content of nitrogenous substances, purines, etc., on the composition of the urine.

The rats were all males of about 150 g. at the beginning of the experiment and the urines were usually collected and analysed every second day. In some

later experiments only two rats were kept in each cage and the urines were collected over a period of four days. The urinary analyses were made by the following methods. Total nitrogen by Kjeldahl, urea by van Slyke's urease method, ammonia by Folin's aeration process, creatinine and uric acid by the Folin colorimetric methods, amino-acids by the formalin method, allantoin by Handovsky's [1914] modification of Wiechowski's technique; it being found more satisfactory to determine nitrogen in the mercury precipitate than to estimate the excess of that element in the final filtrate by volumetric means. Duplicate analyses were made in practically all cases.

Table II. *Diet A. (Control 1.)*

No. of rats	Period of hours	Volume of urine cc.	Total nitrogen g.	Urea-nitrogen		Ammonia-N		Creatinine-N		Uric acid-N		Allantoin-N		Undetermined N	
				g.	% of total N	g.	% of total N	g.	% of total N	g.	% of total N	g.	% of total N	g.	% of total N
4	48	80	2.061	1.801	87.39	0.093	4.5	0.0149	0.7	0.0051	0.28	--	--	0.144	7.13
4	--	267	6.845	6.110	89.09	0.197	1.1	0.0416	0.6	0.0145	0.20	--	--	0.482	5.41
4	96	218	4.922	1.576	32.06	0.105	2.14	0.0446	0.9	0.0125	0.25	0.143	2.9	0.0409	0.85
4	72	185	3.912	3.538	89.74	0.0897	2.21	0.0312	0.8	0.0107	0.27	0.164	4.2	0.1084	2.78
4	96	245	5.995	5.258	87.70	0.1612	2.60	0.0405	0.7	0.0121	0.20	0.100	1.7	0.0232	7.1
Av. daily output per 100 g. rat weight (for last 3 experiments only)			8.51	0.1963	0.1767		0.0047		0.00145		0.00046		0.0051		0.0131

Table III. *Diet E.*

0	48	365	14.460	13.540	93.63	0.4772	3.3	0.0382	0.26	0.0150	0.1	--	--	0.3896	2.71
4	96	410	14.920	14.17	94.95	0.364	2.4	0.0443	0.30	0.0130	0.09	0.138	0.90	0.1907	1.36
4	72	298	10.690	10.01	93.69	0.3142	2.9	0.0274	0.26	0.01	0.09	0.113	1.06	0.2151	2.00
4	96	355	14.01	12.86	91.81	0.4055	2.9	0.035	0.25	0.0125	0.1	0.108	1.16	0.6862	3.79
Av. daily output per 100 g. rat weight (for last 3 experiments only)			15.38	0.5735	0.536		0.0157		0.00155		0.0005		0.0052		0.0199

Table IV. *Diet A "modified." (Control 2.)*

4	120	440	9.363	7.952	84.94	0.282	4.10	0.0193	0.5	0.0201	0.2	0.400	4.3	0.660	5.96
2	96	144	3.377	2.963	87.76	0.131	3.9	0.0226	0.7	0.007	0.2	0.1617	4.8	0.0918	2.64
Av. daily output per 100 g. rat weight			9.9	0.221	0.1913		0.0076		0.0013		0.0005		0.010		0.0083

Table V. *Diet C.*

4	120	475	15.310	13.73	89.69	0.4543	3.7	0.0297	0.2	0.0139	0.09	0.3144	2.1	0.7077	4.22
2	96	174	7.318	6.674	91.20	0.2606	3.6	0.0105	0.27	0.0067	0.09	0.130	1.8	0.2272	3.04
Av. daily output per 100 g. rat weight			15.65	0.5653	0.511		0.0183		0.0013		0.0005		0.0109		0.0233

The results are set out in Tables II to V. In the first place it is of interest to note the diuresis that is marked as a result of the high protein diets. The volume of urine passed daily by the rats on these diets was nearly double

that voided by the controls on the low-protein ration. It seems probable that the diuresis is due to the greatly increased urea formation in the former animals.

Apart from this an inspection of the tables reveals very little. On the diets rich in caseinogen there is the expected rise of urea and ammonia nitrogen; in all cases there is the constancy of creatinine and uric acid excretion that one would anticipate, whilst the allantoin excretion reflects the fate of the purine bases that are added to the diet in the form of the yeast extract.

In other words, these analyses reveal no disturbance of the nitrogenous metabolism that could be regarded as a possible cause of the failing nutrition of the rats fed on diets rich in protein and imperfectly balanced as regards yeast.

*Attempts to determine the nature of substances responsible  
for the effect of yeast.*

In the first place it seemed essential to ascertain whether the action of the yeast extract was attributable to inorganic or organic constituents. A litter of eight young rats was divided into four groups. Two were fed on the high-protein, high-yeast diet (*C*), whilst two others were fed on the high-protein, low-yeast diet (*E*) to which had been added the ash corresponding to the additional 12 parts of yeast extract that were present in diet *C*. The growth curves of these animals are shown in Charts 1 and 2, Fig. 2, and indicate that the salts present in the ash of the other 12 parts of yeast extract did not serve to permit good growth on the high-protein ration. The question then arose as to how it could be determined whether vitamin B was responsible for the effect we were studying. It is generally accepted that this substance is unstable to alkalis and that the antineuritic and growth-promoting properties of foodstuffs such as yeast are not shown by the usual tests after they have been treated with alkalis. Accordingly a diet was constituted in which 70 parts of caseinogen and 4 parts of yeast extract were supplemented with 12 parts of extract that had been autoclaved at 120° for 1 hour at a strongly alkaline reaction and subsequently adjusted to the original hydrogen ion concentration of the extract by the addition of acid. To our surprise this material was, if anything, even more effective in enabling growth on high protein to be satisfactory than the untreated extract (Chart 3, Fig. 2).

The control tests in which all the yeast extract was given in the form of the material that had been treated with alkali (Chart 4, Fig. 2) demonstrated that no growth was made. The nutritive failure of these two animals was checked and growth resumed when the diet was further supplemented with 5 mg. daily of a concentrate prepared from yeast according to Seidell's method [1926].

These experimental results seemed clearly to indicate that at least two



substances, both organic in character, are responsible for the effect of the yeast and that one is probably identical with the substance that is generally known as vitamin B.

The preparation of the yeast concentrate that showed this effect was placed at our disposal by the kindness of Dr A. Seidell. He has described its preparation [1926] and its power of preventing loss of weight in pigeons in

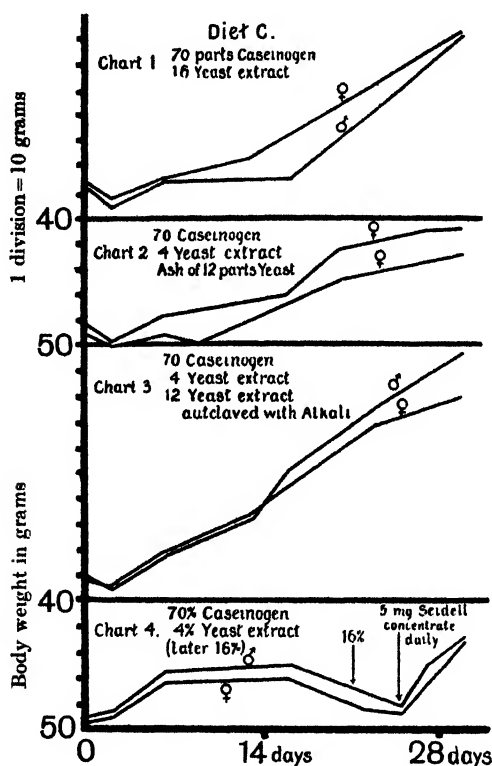


Fig. 2.

doses of about 10 mg. daily. Tested on rats in the usual manner for testing for the presence of vitamin B in foodstuffs we found that the following increments were registered on administering the doses stated:

Dose of yeast concentrate	Change of weight per week
2 mg.	+ 3.5
5 "	+ 5.3
10 "	+ 7.0
20 "	+ 9.7

On the basis that a diet of normal protein content when supplemented with 20 mg. of this concentrate is adequate for steady growth the attempt was made to determine whether increasing the dose of concentrate in proportion to the increase of yeast that had been found necessary would enable good growth to take place on diets rich in protein.

It was found that such was not the case. Doses as high as 80 mg. daily of this preparation did not supplement a diet containing 70 parts of caseinogen as did the equivalent 16 parts of yeast extract.

Convinced from these results that at least two dietary factors derived from the yeast were concerned, we conducted a series of experiments on rats fed on the usual vitamin B-deficient diet employed in the laboratory in order further to trace the relationship between those stable to alkali, and those destroyed by that agent.

The following changes in weight were recorded for a series of pairs of rats of nearly the same initial weight fed on the standard vitamin B-deficient diet with various supplements.

*Average weekly alterations in weight of rats fed on vitamin B-deficient ration plus supplements.*

Alkali-treated	Supplement		Change in g.
	yeast extract % in diet	Seidell's concentrate mg. per day	
	0	0	- 9.0
	0	2	+ 3.5
	0	5	+ 5.5
	2	0	- 7.5
	4	0	- 7.5
	2	2	+ 5.7
	2	5	+ 5.0
	4	2	+ 10.5
	4	5	+ 26.0

The average weekly increment of rats of this age on the basal diet supplemented with 4 % of untreated yeast extract is about 20 g., so that the group receiving 4 % of alkali-treated extract plus 5 mg. of Seidell's concentrate can be regarded as showing a normal rate of growth.

### DISCUSSION<sup>1</sup>.

It seems clear from these results that definite indication has been obtained of the existence of two factors in the yeast extract we employed (marmite) that play an important part in regulating growth in young rats. These factors may be differentiated by the action of hot alkali which destroys one leaving the other still physiologically active. It is seemingly the latter that is primarily concerned in the relation between yeast extract and the protein content of the diet that has been traced.

It is possible that our observations provide an explanation of the good growth on diets rich in protein shown by Osborne and Mendel's rats [1921] which received a ration containing two sources of vitamin B, namely yeast and alfalfa.

<sup>1</sup> When this paper was in the press our attention was called by Dr Seidell to the publications of Goldberger and his colleagues [1928] with which we were unfortunately not familiar. In these evidence is presented of the existence of more than one dietary factor of the vitamin type in yeast. The relation between Goldberger's P-P factor, "bios" and our alkali-stable unit awaits investigation.

They are also obviously closely related to those reported by Seidell [1926] who found that autoclaved yeast, in which, presumably, the antineuritic factor had been largely destroyed, supplemented the growth-promoting effect of his preparation of yeast concentrate in a curious manner and also to those published by Smith and Hendrick [1926].

The question of the identity of the so-called antineuritic vitamin with the water-soluble growth-promoting factor has been many times discussed without anything approaching a definite answer having been obtained.

Perhaps the most convincing work recorded in the literature is that of Funk and Dubin [1922] and Funk and Paton [1922] who drew attention to the fact that the rat requires more than one accessory substance of the vitamin B type for growth and well-being. This conclusion was reached as a result of experiments on the fractional adsorption of substances in yeast extracts on the surface of fuller's earth and norit. They believed these substances to be firstly that usually described as the antineuritic factor, and secondly a substance or group of substances responsible for the so-called "bios" effect observed in the growth of yeasts and other micro-organisms<sup>1</sup>.

It is not proposed in this paper to review the extensive literature on the question of the number and characteristics of the water-soluble vitamins as in our opinion this can only be done adequately when the results of further investigations are to hand.

#### SUMMARY.

1. Evidence is presented that the failure to grow at a normal rate shown by rats fed on diets consisting largely of protein (caseinogen) but containing the usual proportion of yeast serving as a source of vitamin B is not due to an impairment of the digestion or absorption of the ingested protein.

2. No signs of an abnormal course of protein metabolism could be detected in such animals by a study of the distribution of the chief nitrogenous constituents in the urine. The increased urea production on the protein-rich diets causes a marked diuresis.

3. The subnormal rate of growth alluded to is apparently due neither to deficiencies in the inorganic constitution of the ration nor to disturbances of the acid-base exchange.

4. The restoration of a normal rate of growth that follows the addition of extra yeast extract is also seen when the extract has previously been treated with hot alkalis so as to inactivate the factor generally known as vitamin B.

5. For a normal rate of growth to be shown by rats fed on diets consisting mainly of protein two factors, which can be differentiated by their behaviour on treatment with alkalis, seem to be necessary.

<sup>1</sup> Funk gave this factor the designation "vitamin D." Unfortunately at about the same time McCollum suggested the title of vitamin D for the antirachitic vitamin. Some slight confusion followed but has now disappeared as a result of the almost general adoption of McCollum's choice. A satisfactory nomenclature of these water-soluble vitamins will have to be elaborated.

6. One of these would appear to be the substance known as vitamin B or the antineuritic vitamin. It is rapidly destroyed by alkalis. The other resists treatment with alkali and would seem to be the factor chiefly concerned in the action of the yeast supplements in rendering high-protein diets adequate for growth.

We desire to express our thanks to the Medical Research Council for a grant from which part of the expenses of this research were defrayed. The other part was covered by a grant made to one of us (A. H.) by the Egyptian Educational Mission to whom our thanks are also due.

## REFERENCES.

- Drummond, Crowden and Hill (1922). *J. Physiol.* **56**, 413.  
Funk and Dubin (1922). *Proc. Soc. Exp. Biol. Med.* **19**, 15.  
Funk and Paton (1922). *J. Metab. Res.* **1**, 737.  
Goldberger and Lillie (1926). *Pub. Health Rep. May 28th* (Washington).  
Goldberger, Wheeler, Lillie and Rogers (1926, 1). *Pub. Health Rep. Feb. 19th* (Washington).  
Handovsky (1914). *Z. physiol. Chem.* **90**, 211.  
Hartwell (1921). *Biochem. J.* **15**, 140.  
— (1924). *Biochem. J.* **18**, 784.  
— (1925). *Biochem. J.* **19**, 1075.  
McCollum and Simmonds (1925). *The Newer Knowledge of Nutrition* (3rd edition).  
Osborne and Mendel (1921). *Proc. Soc. Exp. Biol. Med.* **18**, 167.  
Reader and Drummond (1926). *Biochem. J.* **20**, 1256.  
Seidell (1926). *Bull. Soc. Chim. Biol.* **8**, 746.  
Smith and Hendrick (1926). *Pub. Health Rep. Feb. 5th* (Washington).

## XC. A CONDUCTIVITY METHOD FOR THE DETERMINATION OF CARBON DIOXIDE.

By LEONARD ERNEST BAYLISS (*Sharpey Scholar*).

*From the Department of Physiology and Biochemistry,  
University College, London.*

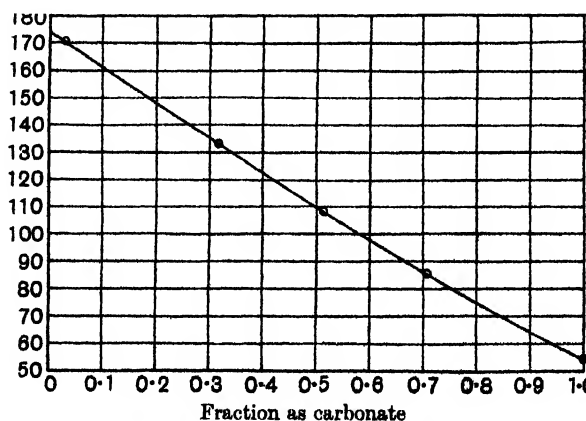
(Received May 1st, 1927.)

BEING faced with the necessity of absorbing some 100 cc. of  $\text{CO}_2$  in 10 minutes and of estimating the quantity absorbed as rapidly as possible and with a possible error not greater than 0.5 %, the usual methods for determining  $\text{CO}_2$  were all examined and found wanting in some respect.

It was necessary, therefore, to devise a new and rapid method of estimating carbonate in a mixture of carbonate and hydroxide, and use was made of the fact that the migration velocity of the  $\text{CO}_3''$  ion is considerably smaller than that of the  $\text{OH}'$  ion, so that the conductivity of a solution of  $\text{NaOH}$  falls as it absorbs  $\text{CO}_2$ .

### *Calibration.*

This is very easily performed, since it is only a matter of plotting a calibration curve showing the conductivities of mixtures, in varying proportions, of solutions of  $\text{NaOH}$  and  $\text{Na}_2\text{CO}_3$  of equivalent concentration. Such a curve is shown in the figure, and it will be seen that it is nearly a straight line for carbonate proportions above 0.3. As points lower than this will practically never be required (the volume of solution used to absorb the  $\text{CO}_2$  can be adjusted so as to avoid them) only a few points are required to determine the curve.



Conductivity of mixtures of 0.997  $N$   $\text{NaOH}$  and 0.997  $N$   $\text{Na}_2\text{CO}_3$  in varying proportions. Ordinates, equivalent conductivity at 24.5°. Abscissae, proportion of  $\text{Na}_2\text{CO}_3$  in mixture.

It is not, of course, necessary to use  $\text{CO}_2$ -free  $\text{NaOH}$  either for calibrating or for absorbing, since the carbonate content can be determined, and hence the true proportion of carbonate in any mixture of the impure  $\text{NaOH}$  with pure  $\text{Na}_2\text{CO}_3$  solution. If  $c$  cc. of sodium carbonate solution be mixed with  $(1 - c)$  cc. of sodium hydroxide solution containing  $\alpha$  equivalents of carbonate per equivalent of total base, then the proportion of carbonate in the mixture is given by

$$\beta = c + \alpha(1 - c).$$

Similarly, if a volume  $v$  cc. of  $\text{CO}_2$  is absorbed by  $b$  cc. of sodium hydroxide solution of total base concentration  $B$ , of which a proportion  $\alpha'$  is carbonate, then the final proportion of carbonate in the mixture is given by

$$\beta' = \frac{\alpha'Bb + v/11 \cdot 15}{Bb}.$$

Hence  $v = (\beta' - \alpha') 11 \cdot 15 Bb$ ,  $\beta'$  being, of course, determined from the measured conductivity by means of the calibration curve.

As a special case of the above equations, we have the possibility that the solution used for absorbing has the same carbonate content as that used for calibrating, and then it can be seen that

$$v = c(1 - \alpha) 11 \cdot 15 Bb.$$

If the method is used for a large number of routine determinations, several samples of caustic soda solution for absorbing will have to be made up; and since it is not easy, or indeed necessary, to ensure that all have exactly the same concentration, a correction will have to be made that will reduce the observed conductivity to that of a  $\text{NaOH-Na}_2\text{CO}_3$  solution of the same proportion of carbonate as that used for absorbing, but of the total base concentration used in plotting the calibration curve.

An empirical equation has been derived experimentally—by measuring the conductivities of  $\text{NaOH-Na}_2\text{CO}_3$  mixtures of various concentrations—which holds for concentrations differing from that of the calibrating solution by less than 20 % and for proportions of carbonate greater than 0.3. If the concentration of the solution used for absorbing  $\text{CO}_2$  is  $B/\gamma$  ( $B$  being that of the solution used for calibrating),  $\lambda$  is the conductivity of the absorbing solution as measured after absorption of  $\text{CO}_2$  and  $\lambda_0$  is the conductivity required, *i.e.* that which the calibrating solution would have if it had the same proportion of carbonate as the absorbing solution, then

$$\lambda_0 = \gamma\lambda \{1 + (1 - \gamma) [0.15 + 0.10 (\beta - 0.03)]\}.$$

In this equation  $c$  can be written instead of  $\beta$  if the initial carbonate content of the alkali solutions is small, as it usually is. The values of the constants have been arrived at experimentally, and are subject to an inaccuracy of about 1 %. There will be further errors if very concentrated solutions are used, since the calibration curve will depart too far from the straight line which the analysis assumes.

We can write  $\lambda_0 = \gamma\lambda$  with an error of less than 0.5 % when

# XCI. THE POSSIBLE SIGNIFICANCE OF HEXOSE-PHOSPHORIC ESTERS IN OSSIFICATION.

## PART VII. THE BONE PHOSPHATASE.

By MARJORIE MARTLAND AND ROBERT ROBISON.

*From the Biochemical Department, the Lister Institute, London.*

*(Received May 2nd, 1927.)*

IN the earlier papers of this series [Robison and Soames, 1924] some account was given of the properties of the phosphatase which is present in ossifying cartilage, bones and teeth, and also in the kidney and intestinal mucosa. In the following we give the results of a more detailed study of some of its properties about which further knowledge was desirable in order to understand the part played by this enzyme and by phosphoric esters in the process of ossification.

### OPTIMUM $p_H$ .

It was previously stated that the optimum  $p_H$  of this enzyme lies on a flat curve between 8.4 and 9.4, the activity being nearly constant between these limits, but falling off rapidly on either side of them. This statement was based on results of 18-hour experiments, but later observations suggested that the flattening of the curve was in part due to the gradual inactivation of the enzyme at the higher  $p_H$  values and that over short periods of time the activity continues to increase with the alkalinity up to a  $p_H$  well above 8.4. More exact investigation of the course of hydrolysis has shown that these suppositions are correct. Fig. 1 shows the rate of hydrolysis of glycerophosphoric ester over a period of 5 hours at  $p_H$  between 7.25 and 9.4. Further measurements, not shown on the chart, were taken at the end of 24, 48 and 166 hours. The  $p_H$  was determined from time to time by a micro-colorimetric method (capillator) on 0.02 cc. liquid withdrawn from the reacting mixture. The addition of indicators to the bulk of the liquid was inadmissible on account of the subsequent colorimetric estimations of inorganic phosphate (Briggs). In this experiment the initial rate increased with the alkalinity up to 9.4, the highest  $p_H$  attempted, but at the end of 24 hours the amount hydrolysed at  $p_H$  9.4 (59.8 %) was almost identical with that at 8.9 (59.1 %).

Fig. 2 shows the inactivation of the bone enzyme in 0.4 % solutions of sodium bicarbonate adjusted to different  $p_H$ . The rate of inactivation increases rapidly with the  $p_H$  above 9.0. At 9.55 over 60 % of the activity was lost in 24 hours.



On the acid side of  $p_H$  6.0 inactivation occurs still more rapidly. In 2 hours at  $38^\circ$  20 % of the activity was destroyed at  $p_H$  5.2, 50 % at  $p_H$  4.2, and 100 % at  $p_H$  3.2.

In Fig. 3, Curve A, the rate of hydrolysis over a short initial period (1 hour) is plotted against the  $p_H$ .

Curve B gives the results of another experiment in which the acid range was more particularly investigated. Here, as in Curve A, a slight increase in the rate of hydrolysis is shown on the acid side of  $p_H$  6.0, and this increase must have been greater but for the partial inactivation of the enzyme during the period of the experiment—in this case 2 hours. Correcting for this loss, Curve B would take approximately the course of the dotted line.

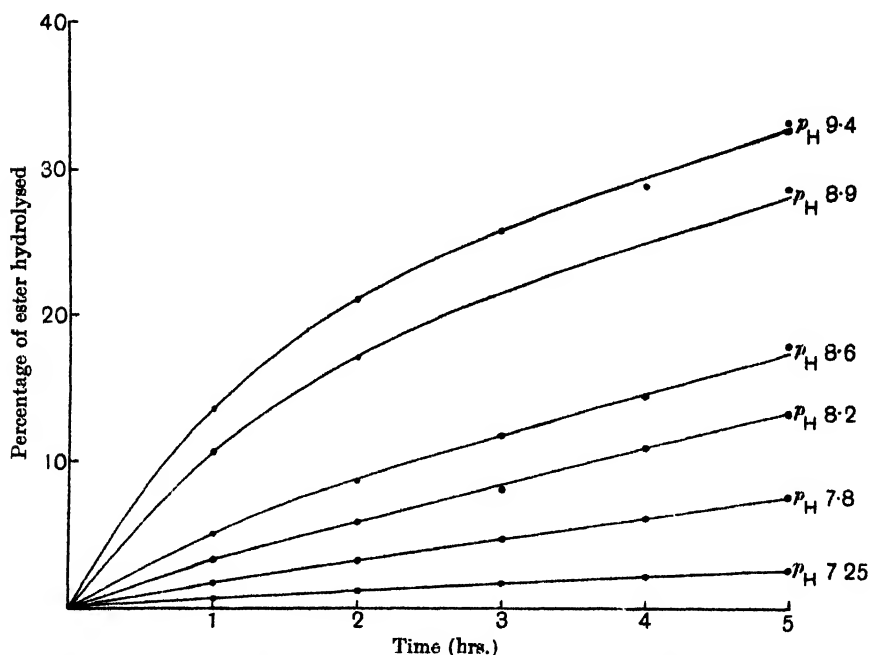


Fig. 1. Hydrolysis of glycerophosphoric ester by bone phosphatase at different  $p_H$ . Concentration of ester 0.1 M. Temp.  $38.0^\circ$ .

Our intention—to measure the initial velocities of the reaction—has not been completely realised in these experiments for, even in their short duration, the retarding effect of the liberated inorganic phosphate (see below) was appreciable, especially at the higher  $p_H$ . The true curve of initial velocities would, therefore, probably show a slightly steeper gradient than that of Curve A.

This activity- $p_H$  curve resembles the dissociation curve of a weak acid, or the dissociation-residue curve of a weak base, and recalls those obtained by Michaelis and his co-workers [1911, 1920] for invertase, except that the slope is in the contrary direction. Michaelis at first explained his results on the assumption that invertase is an acid and that its undissociated molecule

combines with saccharose to form a compound whose decomposition takes place spontaneously as a monomolecular reaction. Subsequent investigation of the relation between the activity- $p_H$  curve and the concentration of the substrate caused him to modify this conception in favour of the hypothesis

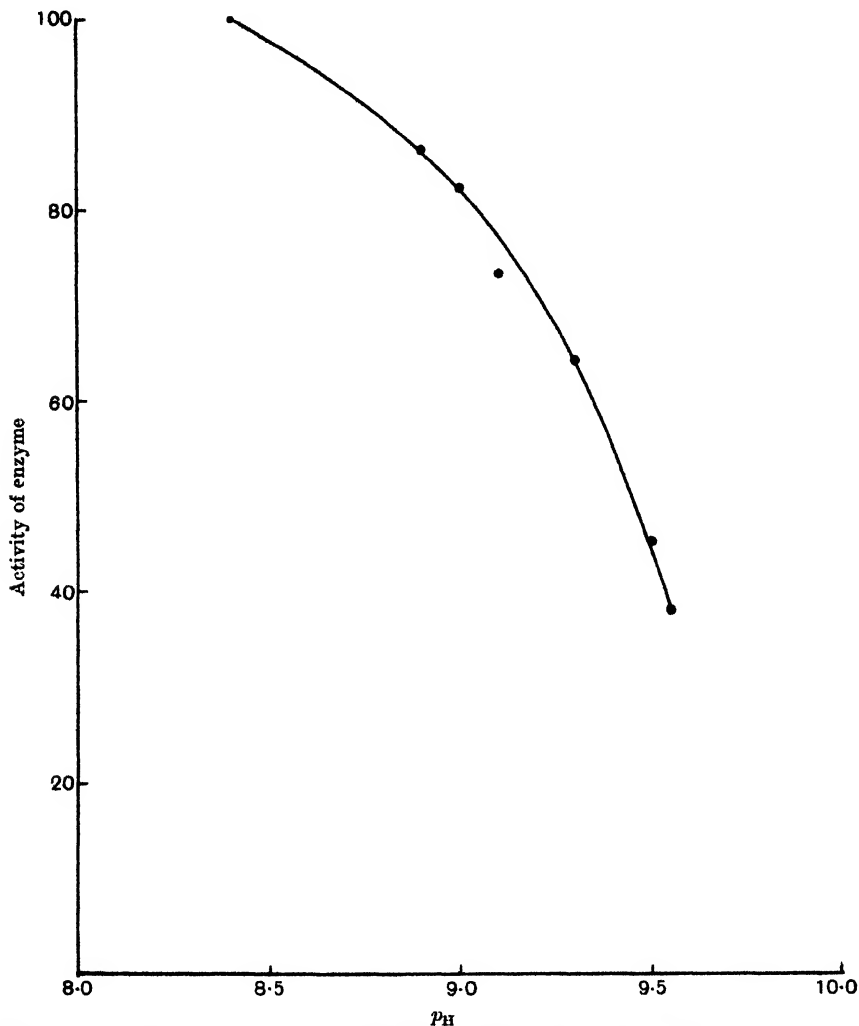


Fig. 2. Inactivation of the bone phosphatase at different  $p_H$ . Bone extract kept 24 hours in 0.4 %  $\text{NaHCO}_3$  solutions adjusted to different  $p_H$ . Temp.  $38.0^\circ$ . Activity of enzyme measured by the hydrolysis of 0.1  $M$  sodium glycerophosphate in  $2\frac{1}{2}$  hours at  $p_H$  9.1, and given as a percentage of the initial activity of the extract.

that invertase combines with the substrate to form a compound with acid properties and that it is the undissociated molecule of this compound which suffers spontaneous decomposition. On this assumption the parameter of the curve is identical with the dissociation constant of the saccharose-invertase acid.

By similar reasoning it may be supposed either (1) that the bone enzyme has the properties of a weak base whose undissociated molecule combines with the phosphoric ester to form a compound which thereupon breaks down into the hydrolysis products of the ester, or (2) that the enzyme combines with the ester to form a compound having the properties of a weak acid, whose anions suffer spontaneous decomposition<sup>1</sup>.

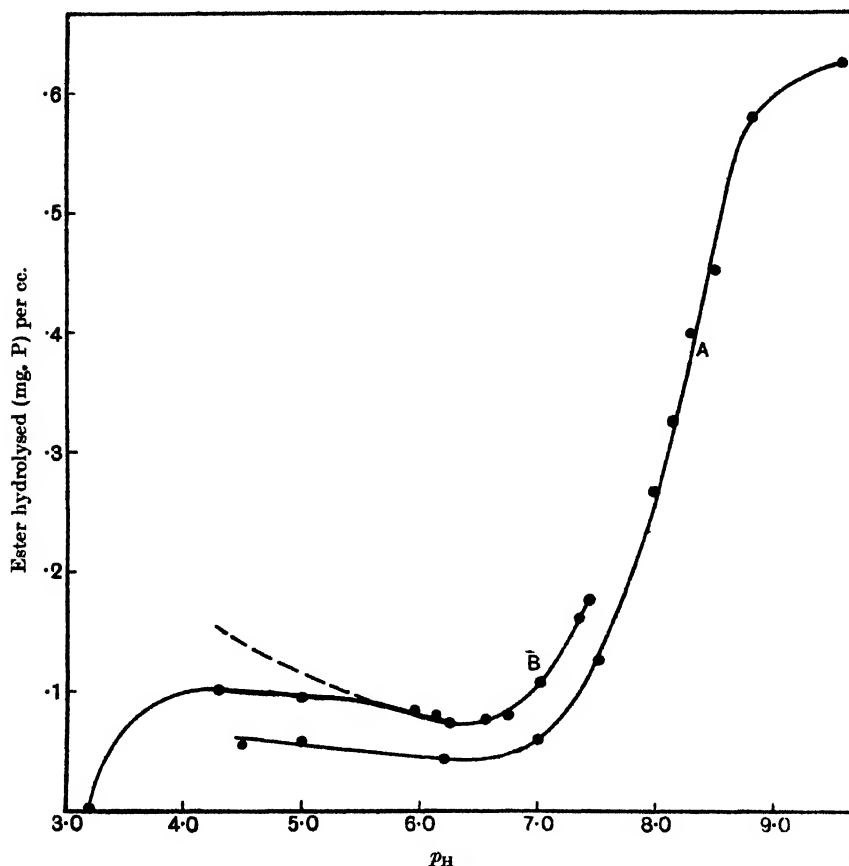


Fig. 3. Activity- $p_H$  curve of the bone phosphatase. Activity measured by the hydrolysis of 0.1 *M* sodium glycerophosphate and given as the amount of ester hydrolysed (mg. P) per cc. in 1 hour. Temp. 39.5°.

Whichever possibility is the correct one, the  $p_K$  of this basic or acidic compound would be equal to the  $p_{OH}$  or  $p_H$  respectively at the point of 50 % dissociation, that is, where the velocity of hydrolysis is half the maximum. The uncertainty connected with the experimental determination of this maximum, which lies in the region where rapid inactivation of the enzyme

<sup>1</sup> There are other possibilities which seem less credible, e.g. that the enzyme-substrate complex has the properties of a base, the concentration of whose undissociated molecule governs the rate of the reaction.

occurs, precludes more than a rough guess at the value of this constant. This appears to lie in the neighbourhood of  $p_H$  8.2.

On the second supposition, the acid would thus be weaker than either glycerophosphoric or phosphoric acid, for which the values of  $p_{K_2}$  are 6.33 and 6.81 [Meyerhof and Suranyi, 1926].

Our present data are insufficient to decide between these alternatives and some doubt must even be admitted as to whether the interpretation of the graph as a dissociation curve is justified. The possibility of some loss of activity occurring even in short periods at  $p_H$  above 9.2 is difficult to eliminate and might account for the flattening of the curve in this region. Further experiments are, however, in progress.

#### EFFECT OF THE CONCENTRATION OF SUBSTRATE ON THE RATE OF REACTION.

Michaelis and Menton [1913] found that the rate of hydrolysis of saccharose by invertase was dependent on the concentration of the substrate and that the "activity- $p_s$ " graph, like that showing the relationship between activity and  $p_H$ , had the character of a dissociation curve.

We have been unable to demonstrate any corresponding variation of activity of the bone phosphatase with concentrations of ester between 0.003  $M$  and 0.3  $M$ . Table I gives the results of two experiments lasting 15 minutes and 1 hour respectively. Such small differences as were observed in the amounts hydrolysed are irregular and cannot be attributed to changes in the substrate concentration.

Table I. *Effect of concentration of substrate on the rate of reaction.*

Concentration of ester (glycerophosphate)	Amount hydrolysed			
	<i>Exp. 1</i>		<i>Exp. 2</i>	
	$p_H$ 8.2. Temp. 38°. Time 15 mins.		$p_H$ 8.5. Temp. 38°. Time 1 hr.	
	mg. P per cc.	% of ester	mg. P per cc.	% of ester
0.3 $M$	0.041	0.45	0.272	3
0.2 $M$	—	—	0.250	4.5
0.1 $M$	0.039	1.3	0.265	9
0.05 $M$	—	—	0.238	16
0.03 $M$	0.043	4.8	0.274	30
0.01 $M$	0.051	17	0.248	80
0.003 $M$	0.039	43	—	—

#### EFFECT OF HYDROLYSIS PRODUCTS ON THE RATE OF THE REACTION.

If the values for  $k = \frac{1}{t} \log \frac{a}{a-x}$ , the velocity constant for a monomolecular reaction, are calculated from the data of hydrolysis experiments such as those quoted on p. 666, these values are found to fall throughout the experiment, and more rapidly than can be accounted for by inactivation of the enzyme. An investigation of the effect of the hydrolysis products on the velocity of the reaction showed that a great part of this retardation is due to the inorganic phosphate, and very little, if any, to the glycerol. Table II gives values of  $k$  for the hydrolysis of sodium glycerophosphate with and

without added inorganic phosphate, showing the effect of varying amounts of the latter on the rate of the reaction. With no added inorganic phosphate the values of  $k$  fall from 0.0133 to 0.0076 during the hydrolysis of 59.8 % of the ester. The initial value of  $k$  is reduced by about 20 % in presence of 0.017  $M$  inorganic phosphate, and by more than 50 % when the concentration of phosphate is increased to 0.033  $M$ . With 0.1  $M$  phosphate the results for the first 5 hours appear to indicate no hydrolysis, and although definite increase of inorganic phosphate was observed after 24 and 48 hours the amount of hydrolysis was less than one-fifth of that found in the control. Results of another experiment with different concentrations of organic and inorganic phosphate are shown graphically in Fig. 4.

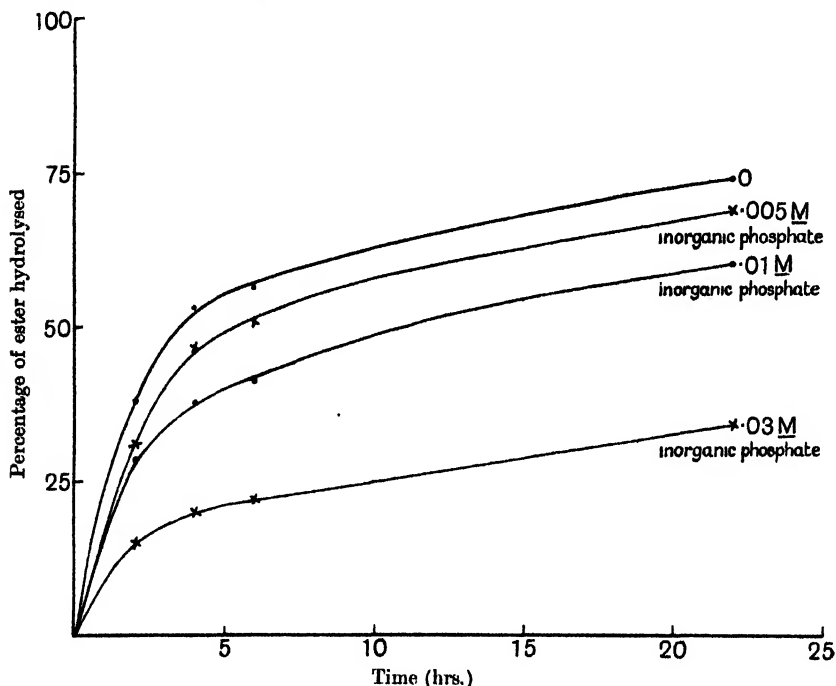


Fig. 4. Effect of inorganic phosphate on the rate of hydrolysis of glycerophosphoric ester by the bone phosphatase. Concentration of ester 0.03  $M$ . Concentration of added inorganic phosphate as shown on each curve. Temp. 38.0°.

Table III gives the results of experiments in which glycerol was added, and shows that this hydrolysis product has very little effect on the rate of the reaction in concentrations up to 0.1  $M$ . The more pronounced effect of glycerol in high concentrations (25 % and over) is discussed in the next section.

#### THE SYNTHETIC ACTION OF THE ENZYME.

The depression of the velocity of hydrolysis by inorganic phosphate observed in the previous experiments suggested that the reverse reaction, synthesis of phosphoric ester, was proceeding simultaneously at an appreciable

rate. We were, however, unable to obtain conclusive evidence of synthesis starting from inorganic phosphate and glycerol in the concentrations (0.01 *M*–0.1 *M*) used in these experiments.

Table II. *Effect of inorganic phosphate on the rate of hydrolysis of glycerophosphoric ester by bone phosphatase.*

Concentration of ester 0.1 *M*, *p*<sub>H</sub> 8.05, temp. 37°.

Concentration of added inorganic phosphate as shown below.

Hrs.	Inorg. P = 0			Inorg. P = 0.017 <i>M</i>			Inorg. P = 0.033 <i>M</i>			Inorg. P = 0.1 <i>M</i>		
	% of ester hydrolysed	$\frac{1}{t} \log \frac{a}{a-x}$	$k$	% of ester hydrolysed	$\frac{1}{t} \log \frac{a}{a-x}$	$k$	% of ester hydrolysed	$\frac{1}{t} \log \frac{a}{a-x}$	$k$	% of ester hydrolysed	$\frac{1}{t} \log \frac{a}{a-x}$	$k$
1	3.02		0.0133	2.48		0.0109	1.28		0.0051	0		0
2	5.67		0.0121	4.62		0.0097	2.67		0.0061	0		0
5	12.11		0.0103	9.17		0.0071	4.98		0.0035	0		0
24	38.97		0.0083	34.05		0.0062	23.58		0.0050	7.73		0.0015
48	59.80		0.0076	50.38		0.0060	32.87		0.0024	11.77		0.0008

Table III. *Effect of glycerol on the rate of hydrolysis of glycerophosphoric ester by bone phosphatase.*

Concentration of ester 0.1 *M*, *p*<sub>H</sub> 8.4, temp. 38°.

Concentration of added glycerol as shown below.

Hrs.	Percentage of ester hydrolysed		
	Glycerol = 0	Glycerol = 0.01 <i>M</i>	Glycerol = 0.1 <i>M</i>
1	9.7	9.7	9.2
2	15.3	17.5	16.5
5	30.3	31.3	32.2

When the concentration of the glycerol was considerably increased (and the active mass of the water thereby reduced) the synthetic action of the enzyme became very evident, up to 25 % of the inorganic phosphate being converted into phosphoric ester. Synthesis of phosphoric esters was also observed when glycerol was replaced by other polyhydric alcohols, glycol, mannitol, glucose and fructose, but in 50 % ethyl alcohol neither synthesis nor hydrolysis (of glycerophosphate) occurred to an appreciable extent.

The experiments were carried out by adding the alcohols and solid  $K_2HPO_4$  to the bone extract and keeping the mixture at 38°, withdrawing 0.2 cc. into 10 cc. 3 % trichloroacetic acid at intervals. Duplicate determinations of total P and inorganic phosphate were carried out on the protein-free filtrates. No attempt was made to determine the *p*<sub>H</sub> of these concentrated solutions, but the presence of the alkaline phosphate was relied on to maintain a suitable reaction.

The results of some of the experiments are set out in Table IV, the amount of phosphoric ester synthesised being calculated as a percentage of the inorganic phosphate added.

Table IV. *Synthesis of phosphoric esters by the bone phosphatase.*

	Alcohol	Concentration of $K_2HPO_4$	Amount of P-ester formed calculated as % of inorganic P added				
			1 day	7 days	14 days	21 days	28 days
Glycerol 50 % by vol.	...	0.07	6.0	22.7	—	24.5	—
" "	...	0.2	1.8	12.3	19.3	—	22.6
" "	...	0.5	0.8	3.9	5.4	—	10.6
" 66 %	...	0.12	—	7.0	12.3	—	16.4
Glycol 25 % by vol.	...	0.1	3.8	11.9	17.3	—	16.3
" 50 %	...	0.1	6.9	17.2	22.4	23.1	—
" "	...	0.1	—	18.3	24.8	23.5	—
Alcohol 50 %	...	0.1	0	0	—	—	—
Mannitol, sat. solution, about 30 %	...	0.16	—	8.5	9.8	8.3	—
Glucose 60 %	...	0.12	1.8	2.8	5.8	5.3	—
Fructose 60 %	...	0.12	—	3 days 4.0	—	—	—

THE EFFECT OF HORMONES ON THE RATE OF HYDROLYSIS AND OPTIMUM  $p_H$  OF THE ENZYME.

In view of the work of Langfeld [1921], who claimed to have demonstrated that the optimum  $p_H$  of liver diastase was shifted in presence of various glandular extracts, a series of experiments was performed to find whether such an effect could be obtained with the bone phosphatase. The relative rates of hydrolysis were measured at various  $p_H$  ranging from 7.0 to 9.3 with additions of the following hormones.

Adrenaline	$\frac{1}{25,000} - \frac{1}{2,500,000}$	(dilution in total volume)
Pituitrin	$\frac{1}{25} - \frac{1}{2,500}$	( " " )
Thyroid	$\frac{1}{400} - \frac{1}{40,000}$	( " " )

It is not necessary to give in detail the results of the numerous experiments carried out, but in no case was any definite effect detected on the rate of hydrolysis or the optimum  $p_H$ . Similar negative results have also been reported by Demuth [1925].

THE ACTION OF THE BONE PHOSPHATASE ON VARIOUS PHOSPHORIC ESTERS.

Table V shows the action of the bone phosphatase on mono- and diethyl phosphoric esters, which were kindly given to us by Prof. R. H. A. Plimmer and Mr W. J. N. Burch. The enzyme had very little action on the diethyl ester but the monoethyl ester was readily hydrolysed. The enzyme was also found to be without action on a fully methylated derivative of hexosediphosphate prepared by Mr W. T. J. Morgan [1927] whereas the methylhexosediphosphate was readily hydrolysed. These differences may perhaps be attributed simply to the steric hindrance of the adjacent methyl or ethyl groups, but in view of the much greater affinity of the enzyme for inorganic phosphate than for phosphoric esters, and its very small affinity for glycerol, it is an interesting speculation as to whether the combination of

enzyme and substrate takes place through the hydroxyl groups attached to the phosphorus, the presence of at least two such groups being required.

Table V. *Action of bone phosphatase on mono- and di-ethyl phosphates.*

Ester	Percentage of ester hydrolysed Hours					
	1	2	3	5	24	48
Potassium monoethyl phosphate 0.05 <i>M</i>	8.0	11.7	18.9	26	57	69
Potassium diethyl phosphate 0.08 <i>M</i>	0.1	0.3	0.3	0.4	0.5	0.7

The discovery of the marked effect of inorganic phosphate on the rate of hydrolysis furnished an explanation of a difficulty encountered in attempting to use the bone phosphatase for the hydrolysis of hexose mono- and di-phosphoric esters and the investigation of their sugar component.

When the ester was used in such concentration as would give a reasonable amount of the sugar, hydrolysis rapidly came to an end, although the enzyme was shown to be still active. The difficulty was overcome by carrying out the hydrolysis in presence of barium ions by which the free phosphate was precipitated in the form of  $\text{Ba}_3(\text{PO}_4)_2$ . As the formation of this salt involved a fall in the  $p_{\text{H}}$  sufficient baryta was added at frequent intervals to maintain a  $p_{\text{H}}$  of about 8.4 to 8.8. In this way quantities of 0.5 g. barium hexosediphosphate can be almost completely hydrolysed in a few days. The drawback of this method for use with the free hexosephosphoric esters lies in the necessary use of an alkaline reaction and the consequent intramolecular transformation of the sugars which may be anticipated. This objection does not apply to methylhexosides of these hexosephosphates, and the application of this method to these compounds may furnish useful information as to the nature of the sugar [v. Morgan, 1927]. The method is in fact analogous to the case of calcification in the body where any increase of inorganic phosphate in the cartilage due to the activity of the enzyme would normally be followed by the precipitation of this excess as calcium phosphate, leaving the activity of the enzyme unimpaired.

#### SUMMARY AND CONCLUSIONS.

1. The activity of the bone phosphatase increases with the alkalinity up to  $p_{\text{H}}$  9.4, but above 8.4 this is offset by the gradual inactivation of the enzyme.

In the body the optimum  $p_{\text{H}}$  may therefore be taken as about 8.4.

2. The activity- $p_{\text{H}}$  curve has the form of the dissociation curve of a weak acid,  $p_{\text{K}}$  about 8.2, or the dissociation-residue curve of a weak base.

3. Variations in the concentration of the substrate between 0.003 *M* and 0.3 *M* do not affect the initial rate of hydrolysis.

4. The rate of hydrolysis of glycerophosphoric ester is greatly retarded by inorganic phosphate even in low concentrations, but is not appreciably affected by similar concentrations of the second hydrolysis product—glycerol.



5. In high concentrations of glycerol and in presence of inorganic phosphate, synthesis of phosphoric ester takes place, as much as 25 % of the inorganic phosphate being esterified under certain conditions.

6. The synthetic action of the enzyme has likewise been demonstrated with mannitol, glycol, glucose and fructose.

7. No effect on the rate of hydrolysis or the optimum  $p_H$  of the enzyme has been detected as a result of adding adrenaline, pituitrin or thyroid.

8. The action of the enzyme on various types of phosphoric ester is discussed.

#### REFERENCES.

- Demuth (1925). *Biochem. Z.* **166**, 162.  
Langfeld (1921). *J. Biol. Chem.* **46**, 391.  
Meyerhof and Suranyi (1926). *Biochem. Z.* **178**, 427.  
Michaelis and Davidsohn (1911). *Biochem. Z.* **35**, 386.  
Michaelis and Menton (1913). *Biochem. Z.* **49**, 333.  
Michaelis and Rothstein (1920). *Biochem. Z.* **110**, 217.  
Morgan (1927). *Biochem. J.* **21**, 675.  
Robison and Soames (1924). *Biochem. J.* **18**, 740.

## XCII. THE CHEMISTRY OF HEXOSE-DIPHOSPHORIC ACID. PART I.

### THE $\alpha$ - AND $\beta$ - METHYLHEXOSIDEDIPHOSPHORIC ACIDS<sup>1</sup>.

By WALTER THOMAS JAMES MORGAN

(*Grocers' Company Research Scholar*).

*From the Biochemical Department, Lister Institute, London.*

(Received May 2nd, 1927.)

THE application of the process of methylation in the sugar group has proved of such great value in the elucidation of the structure of these compounds that it seemed to afford a promising method of investigating the constitution of hexosediphosphoric acid.

The general problem of the chemical constitution of hexosediphosphoric acid divides itself naturally into two parts. The first concerns the exact nature of the hexose portion of the molecule, while the second deals with the position of the two phosphoric acid groups in the six carbon atom chain.

The earlier work of Young [1909] showed that hexosediphosphoric acid on hydrolysis with boiling hydrochloric acid yielded free phosphoric acid along with a laevo-rotatory solution containing fructose or possibly some other reducing sugar. The fact observed by Young that the ratio of the reducing power to the rotation of the product of hydrolysis was greater than corresponds with pure fructose was explained by Neuberg and Kretschmer [1911] as due to the formation of decomposition products owing to the prolonged boiling with acid. That this explanation was not in itself sufficient was shown later by Young who found that pure fructose after heating with phosphoric acid for some hours showed only a slight decrease in the ratio  $\frac{\text{Rotation}}{\text{Reduction}}$ .

The behaviour of hexosediphosphoric acid towards phenylhydrazine has been investigated both by Lebedev [1909, 1910, 1911] and Young [1911]. The latter worker found that a molecule of phosphoric acid was split off during the formation of the osazone, but that, if the reaction was carried out, using cold reagents, hexosediphosphoric acid reacts without loss of phosphorus to form a derivative which he regarded as a phenylhydrazine salt of hexosediphosphoric acid phenylhydrazone. These reactions led Young [1911] to put forward the following structures which show the probable position of one of the phosphoric acid groups in the molecule. Formula I assumes glucose and formula II fructose as the parent hexose in hexosediphosphoric acid.

<sup>1</sup> This paper was communicated to the Biochemical Society on Feb. 7th, 1927, and an abstract was published in *Chemistry and Industry*, 1927, 46, 129 (Feb. 11th).



The production of a fully methylated derivative of hexosediphosphoric acid offers a fresh method of attacking the constitution of this substance, since it has been shown by the work of Purdie and Paul [1907], Irvine and Patterson [1922] and Howarth and Hirst [1926] that the methylation and subsequent oxidation of fructose has yielded sufficient evidence for the determination of the constitution of this sugar.

Some early experiments on the production of a methylated hexose-diphosphoric acid showed that considerable oxidation occurred during the methylation. When the silver oxide was added to the hexosediphosphoric acid dissolved in methyl alcohol and methyl iodide, a vigorous reaction ensued, but only a small yield of the methylated compound was obtained. Even when the alkylating agent was used in considerable excess, the methyl derivative was produced in very small quantity. As has been explained by Purdie and Irvine [1903] in the case of glucose, the poor yield is partly due to the fact that the alkylation extends to the solvent methyl alcohol and that much of the alkylating agent is thus lost. Examination of the silver residues after the methylation showed that there had been a production of metallic silver, thus indicating that oxidation had occurred. Previous work on the production of tetramethylglucose and tetramethylfructose showed that to avoid oxidation during methylation it was necessary that the aldehydic or ketonic group of the sugar should be protected, and that instead of the free sugar, the methylglucoside or methylfructoside should be used. The application of these principles to hexosediphosphoric acid has led to the preparation of the methyl-hexosedidiphosphates.

Since hexosediphosphoric acid is a derivative of a hexose, most probably fructose, it must necessarily, from stereochemical considerations, exist in  $\alpha$ - and  $\beta$ -modifications, corresponding to the  $\alpha$ - and  $\beta$ -forms of the parent sugar. Obtained as a result of yeast fermentation, hexosediphosphoric acid has an optical activity,  $[\alpha]_D + 3.4^\circ$ , which suggests that it is a mixture of two isomeric acids. In order to detect the production of the two methyl-hexosedidiphosphoric acids during the condensation of hexosediphosphoric acid with methyl alcohol, this process, and subsequent hydrolysis of the hexoside, were followed by polarimetric observations.

Assuming that hexosediphosphoric acid is a derivative of the ordinary *d*-fructose, the methylhexosedidiphosphate corresponding to  $\beta$ -methyl-*d*-fructoside,  $[\alpha]_D - 172^\circ$ , should possess the greater laevo-rotation of the two hexosides.  $\alpha$ -Methylfructoside,  $[\alpha]_D - 10^\circ$ , is much less laevo-rotatory than the corresponding  $\beta$ -compound, that is, the more strongly dextro- or less laevo-rotatory methylhexosedidiphosphate should correspond to  $\alpha$ -methylfructoside and would itself be  $\alpha$ -methylhexosedidiphosphate. Since the methylhexoside-

diphosphate as obtained by Fischer's method is dextro-rotatory, one component of the mixture must possess a dextro-rotation and this, from the above reasoning, would be the  $\alpha$ -methylhexosidediphosphate. It has been shown by Purdie and Paul [1907] that  $\alpha$ -methylfructoside ( $\beta$ -methylfructoside in their paper) is more rapidly formed than the corresponding  $\beta$ -compound when *d*-fructose is methylated by Fischer's method. Applying these considerations to hexosediphosphoric acid, it would be expected that the  $\alpha$ -methylhexosidediphosphate would be formed more rapidly at the commencement of the experiment and thus give rise to an increase in dextro-rotation; the slower production of the  $\beta$ -compound causing the optical rotation of the solution to fall towards the end of the experiment. Exactly the reverse of these optical changes should take place on complete hydrolysis of the mixed methylhexosidediphosphates by acid. There should be a fall in optical rotation, corresponding with the more rapid hydrolysis of the  $\alpha$ -compound, followed by a diminished negative rotation towards the end of the hydrolysis. It will be seen from the experimental portion of this paper (Figs. 1 and 2) that there is complete qualitative correspondence between the predicted and experimental changes in optical rotation.

*Preparation of barium methylhexosidediphosphate.*

The hexoside was prepared from pure barium hexosediphosphate according to Fischer's method [1895]. The methyl alcohol used in all the experiments was free from acetone [see Menzies, 1922] and was fractionally distilled over sodium immediately before use, the portion boiling between 64.5° and 65.5° being collected.

The barium hexosediphosphate (20 g.) was added in the form of a fine powder to 400 cc. of methyl alcohol containing 6.75 g. of dry hydrogen chloride. These quantities give, after complete solution of the barium salt, a solution containing 0.5 % of free hydrogen chloride, which is the concentration of acid used by Fischer throughout his preparations of the  $\alpha$ - and  $\beta$ -methylglucosides. In order to obtain the barium hexosediphosphate in solution as rapidly as possible the reaction was carried out in a thick glass bottle containing a number of glass beads which served to grind up any large particles of the salt formed during the rapid addition of the barium salt to the acid methyl alcohol. Under these conditions 5 or 6 minutes' shaking completed the solution of the salt and a clear pale yellow methyl alcohol solution resulted. As soon as this stage was reached a portion of the solution was filtered into a 4 dm. polarimeter tube and the rotation measured. If the precautions mentioned were observed it was possible to obtain a polarimeter reading 12–15 minutes after the barium hexosediphosphate had first come into contact with the acid methyl alcohol. The optical rotation, reducing power and inorganic phosphorus of the solution were determined at intervals until methylation was complete. The values obtained for one particular preparation are shown in Table I.

For the first 2 or 3 hours the solution of the barium hexosediphosphate in acid methyl alcohol remained perfectly clear. The barium chloride after this time was rapidly thrown out of solution as a crystalline mass, only about half of the barium, however, being deposited as chloride during the course of the methylation. During the methylation the temperature was kept at 25° since higher temperatures caused a certain degree of hydrolysis of one of the phosphoric acid groups.

Table I.

Time		Observed* rotation	Total phosphorus g.	Inorganic phosphorus g.	Sugar as glucose per 1 cc. of solution mg.
15 mins.		+0.70°	2.01	0.28	5.29
1 hour	5 mins.	+0.74	—	—	—
4 hours	30 "	+0.78	—	—	—
†7 "	30 "	+0.83	2.16	0.31	2.64
24 "		+0.88	—	—	1.80
32 "		+0.79	—	—	—
48 "		+0.66	—	—	—
56 "		+0.57	—	—	—
70 "		+0.54	—	—	1.12
78 "		+0.54	—	—	—
92 "		+0.53	—	—	0.70
100 "		+0.53	2.11	0.37	0.51
114 "		+0.53	1.96	0.34	0.48
144 "		+0.53	—	—	—
168 "		+0.525	2.02	0.33	—

\* Mercury green line.

† Temperature raised to 25° until methylation was complete.

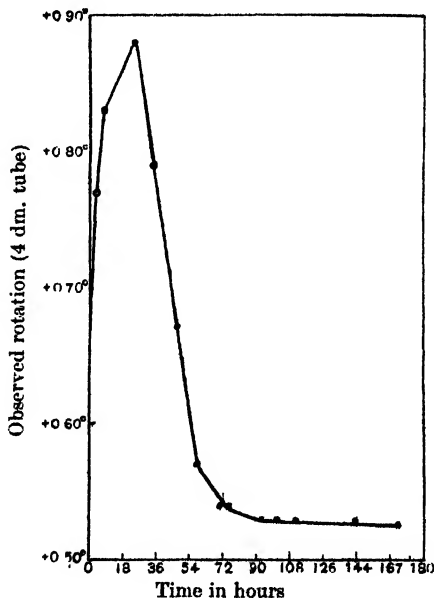


Fig. 1. Curve showing the change in optical rotation during the methylation of hexosediphosphoric acid.

It will be seen from the curve that there is a definite rise followed by a fall in the optical rotation. Since theoretically hexosediphosphoric acid can exist in  $\alpha$ - and  $\beta$ -modifications, the rise and fall in optical rotation at once suggest the methylation of two compounds, one of which methylates to give a hexoside with a more strongly positive rotation than barium hexosediphosphate, while, more slowly, a hexoside with a negative rotation is formed. On arresting the reaction after 24 hours, that is, when the maximum positive rotation was obtained, a methylhexosidediphosphate was recovered which possessed a specific rotation of  $+8.3^\circ$  which is more than twice the value of the original barium hexosediphosphate.

Table I shows that during methylation there was a small but definite increase in the inorganic phosphorus showing that slight hydrolysis of one of the phosphoric acid groups of the hexosediphosphoric acid had taken place. In order to be certain that any phosphorus hydrolysed was not converted to methyl phosphate, thus escaping detection as inorganic phosphate, an experiment was made using orthophosphoric acid in methyl alcohol and hydrogen chloride, the conditions of concentration, time and temperature being similar to the main hexoside preparation. No change, however, in the concentration of inorganic phosphate could be detected.

When the hexoside preparation showed no further fall in optical rotation and caused no reduction of Fehling's solution, the barium chloride formed during the methylation was filtered off and the clear methyl alcoholic solution was shaken with a slight excess of barium carbonate in order to remove the excess hydrogen chloride. The solution was again filtered and neutralised to phenolphthalein with a methyl alcoholic solution of baryta. The crude hexoside was precipitated under these conditions as a white amorphous powder.

*Separation of the  $\alpha$ - and  $\beta$ -barium methylhexosidediphosphates.*

*Fractionation by alcohol.* The crude barium salt, which contained small quantities of barium phosphate, unchanged barium hexosediphosphate and barium chloride, was ground with 5 parts of ice-cold water for about 30 minutes. The mixture was then filtered and the residue again ground with the same quantity of water. This extraction operation was continued until 20 or 25 parts of water had been used. The residue still contained a small amount of organic phosphorus, the last traces of which were exceedingly difficult to remove by extraction with water. The combined filtrates were then fractionally precipitated by the addition of ethyl alcohol.

For the purpose of following the fractionation, the details are given of one particular experiment in which 40 g. of crude, air-dried barium hexosidediphosphate were used.

		Weight of fraction g.
A.	1st fraction. Final alcohol concentration 15 % (by volume)	16.0
B.	2nd " Alcohol concentration increased from 15-35 %	7.7
C.	3rd " " " to about 80 %	5.5

The first fraction (A) was dissolved in 10 parts water and increasing quantities of alcohol were added, when the following sub-fractions were obtained:

	$[\alpha]_{\text{Hg green}}^{18-19^\circ}$	Weight of fraction g.
(1) Fraction precipitated with alcohol up to 5 % concentration	+ 3.0°	5.0
(2) " " increasing from 5-10 % conc.	+ 0.3	5.8
(3) " " " 10-15 % "	- 1.3	1.8
(4) " " " 15-70 % "	- 2.4	1.8

The result of this fractionation showed at once that at least two compounds, one possessing a positive and the other a negative rotation, were present in the crude hexoside preparation.

The second main fraction (B), obtained by increasing the alcohol concentration from 15 to 35 % as shown above, was also dissolved in 10 parts of water and fractionally precipitated with alcohol.

	$[\alpha]_{\text{Hg green}}^{18-19^\circ}$	Weight of fraction g.
(1) Fraction precipitated with alcohol up to 10 % concentration	- 1.1°	3.6
(2) " " increasing from 10-15 % conc.	- 1.9	2.1
(3) " " " 15-35 % "	Slightly positive	About 1 g.

The third main fraction (C), which was only precipitated by alcohol above 35 % concentration, dissolved readily in 10 parts of 20 % alcohol, thus differing considerably in solubility from the first and second fractions. The process of fractional precipitation was carried out as for the previous two fractions but no indication of separation into two different optically active substances was obtained. The specific rotation of the fractions remained constant at about + 0.7°.

The combined fractions from the first main fraction, which possessed a positive rotation, were again dissolved in 10 parts of water and fractionally precipitated with alcohol, the progress of the separation being followed by the determination of the specific rotation of the various fractions. As a result of several fractionations a barium hexosidediphosphate was obtained which possessed a specific rotation of + 4.6°.

Similar methods of separation were then applied to those fractions which possessed negative rotation, when a barium salt possessing a specific rotation of - 2.3° was obtained.

The occasional occurrence of fractions possessing higher specific rotations for example, values such as - 5.5° or + 5.0°, at once suggested that the resolution into optical isomers was far from complete.

*Fractionation by brucine.* The optically active fractions obtained were then converted into brucine salts. The barium salt was dissolved in water and a slight excess of the theoretical quantity of sulphuric acid carefully added in the form of a 2 N solution. Brucine in 10-20 % excess of the calculated quantity was then added in a warm 50 % alcoholic solution, the mixed solutions possessing a pale yellow colour.

In the case of the brucine salt prepared from the  $\beta$ -methylhexoside-diphosphate crystals separated within a few minutes of mixing the solutions. After standing overnight, the crystals were filtered off, dissolved in 10 parts of hot 50 % alcohol and allowed to crystallise. The process of recrystallisation was carried out several times, the mother-liquors from each recrystallisation being concentrated to about half their original bulk and mixed together.

A portion of the sparingly soluble salt so obtained was dried to a constant weight at 78° and 15–20 mm. pressure over phosphorus pentoxide, no discoloration taking place.

Phosphorus, estimated by modified Briggs-Doisy method	Found P 3.1 %
Calculated for $(C_6H_9O_3(PO_4H_2)_2(OCH_3)(C_{23}H_{28}N_2O_4)_4$	P 3.2 %
Specific rotation of the brucine salt in 10 % alcohol (conc. 0.38 %)	$[\alpha]_{H_2O}^{18^\circ}$ green -38.4°

The isolation of the isomeric  $\alpha$ -brucine salt in a pure condition proved a much more difficult process. The preparation of the brucine salt was carried out exactly as has been described above for the  $\beta$ -compound. The  $\beta$ -compound present could be slowly removed by concentrating the alcoholic solution of brucine salt and allowing it to stand in the cold room (0°) overnight. The  $\alpha$ -compound remained in solution. A micro-crystalline mass of the brucine  $\alpha$ -methylhexosidediphosphate could, however, be obtained by pouring this concentrated alcoholic solution into 3 volumes of dry acetone. Under these conditions the excess of brucine remained in solution. This process of acetone precipitation was repeated several times until the brucine salt was free from excess of brucine. It was then fractionally crystallised from hot 96 % alcohol, the process of separation being followed by converting small portions of the fractions into barium salts and determining the specific rotation. The final brucine salt, after drying at 78° (20 mm.) over phosphorus pentoxide, gave the following analytical figures:

Found	P 2.9 %
Calculated for $(C_6H_9O_3(PO_4H_2)_2(OCH_3)(C_{23}H_{28}N_2O_4)_4$	P 3.2 %

#### *Barium $\beta$ -methylhexosidediphosphate.*

The brucine  $\beta$ -methylhexosidediphosphate was converted into the barium salt by treatment with a 50 % alcoholic solution of baryta until the mixture was pink to phenolphthalein. The liberated brucine passed into solution while the barium  $\beta$ -methylhexosidediphosphate was precipitated. It was filtered off, washed with warm 96 % alcohol to remove any adhering brucine, and dried in a vacuum desiccator over sulphuric acid. The salt was then dissolved in 10 parts of ice-cold water and filtered to remove any barium carbonate, the barium salt being again precipitated with alcohol and dried. This process of solution in water and reprecipitation with alcohol must be carried out until the barium salt is perfectly free from brucine, otherwise the observed specific rotation will be much more laevo than the true value.

The final barium salt was obtained as a white amorphous substance which was distinctly more soluble in cold than hot water, thus resembling in solubility the original barium hexosediphosphate.



*Analysis.* For analysis the salt was dried to constant weight over phosphorus pentoxide. After heating for 2 to 3 hours at 100° (20 mm.) it remained perfectly white, in this respect differing from hexose mono- and di-phosphate.

The estimations of methoxyl groups and barium were carried out by Pregl's micro-methods.

	OCH <sub>3</sub>	P	Ba
Found	4.93 %	9.76 %	43.75 %
Calculated for C <sub>6</sub> H <sub>9</sub> O <sub>8</sub> (OCH <sub>3</sub> ) (PO <sub>4</sub> Ba) <sub>2</sub>	4.97 %	9.92 %	43.91 %

Specific rotation of the salt in water (conc. 3.16 %)  $[\alpha]_{\text{Hg green}}^{18^\circ} - 10.4^\circ$

The specific rotation of the  $\beta$ -methylhexosidediphosphoric acid (conc. 1.66 %):

$$[\alpha]_{\text{Hg green}}^{18^\circ} - 23.2^\circ$$

Molecular weight determination by the cryoscopic method yielded the following results, samples from two distinct preparations being used:

(1) 0.0942 g. barium salt in 9.95 g. water gave  $\Delta = -0.074^\circ$ , whence apparent molecular weight = 236

(2) 0.0807 g. barium salt in 9.95 g. water gave  $\Delta = -0.053^\circ$ , whence apparent molecular weight = 284

C<sub>6</sub>H<sub>9</sub>O<sub>8</sub>(OCH<sub>3</sub>) (PO<sub>4</sub>Ba)<sub>2</sub> requires molecular weight = 624; if 100 % dissociated apparent molecular weight = 208

The experimental results are, therefore, consistent with this formula.

The free  $\beta$ -methylhexosidediphosphoric acid, liberated by the addition of the theoretical quantity of sulphuric acid to the pure barium salt, decomposed rapidly. Concentration of the aqueous solution of the acid in a vacuum desiccator led to rapid elimination of the methyl group. By working with ice-cold solutions, it was possible to obtain the acid as a colourless syrup free from inorganic phosphate, but the aqueous solution of this syrup, after standing at room temperature for 36 hours, showed a considerable reducing power, the estimation of which showed that 23 % of the methyl group had been removed.

#### *Barium $\alpha$ -methylhexosidediphosphate.*

This salt was prepared by exactly the same method as has been described for the  $\beta$ -compound and was analysed in the same way.

#### *Analysis.*

	OCH <sub>3</sub>	P	Ba
Found	5.11 %	9.89 %	43.55 %
Calculated for C <sub>6</sub> H <sub>9</sub> O <sub>8</sub> (OCH <sub>3</sub> ) (PO <sub>4</sub> Ba) <sub>2</sub>	4.97 %	9.92 %	43.91 %

Specific rotation of the salt in water (conc. 3.65 %)  $[\alpha]_{\text{Hg green}}^{18^\circ} + 8.2^\circ$

Specific rotation of the free  $\alpha$ -acid (conc. 1.96 %)  $[\alpha]_{\text{Hg green}}^{19.5^\circ} + 19.7^\circ$

Molecular weight determination by the cryoscopic method yielded the following results:

(I) 0.0780 g. barium salt in 9.95 g. water gave  $\Delta = -0.062^\circ$ , whence apparent molecular weight = 235

(II) 0.0557 g. barium salt in 9.95 g. water gave  $\Delta = -0.054^\circ$ , whence apparent molecular weight = 193

*Barium methylhexosidemonophosphate.*

During the preparation of the  $\alpha$ - and  $\beta$ -methylhexosidediphosphates by Fischer's method, there always occurred a slight increase in the inorganic phosphorus present, thus indicating that hydrolysis of the phosphoric acid groups in the original hexosidediphosphoric acid had occurred. The actual amount of inorganic phosphorus so formed varied slightly in each experiment but never exceeded 3.0 % of the total organic phosphorus.

Since one phosphoric acid group is removed by hydrolysis much more readily than the other in hexosidediphosphoric acid, it would seem that a hexosemonophosphoric acid, or methylhexosidemonophosphoric acid as it would become after methylation, would be the most likely product. From the crude barium methylhexosidediphosphates a very soluble barium compound was isolated and proved to be a methylhexosidemonophosphate.

As has already been stated (p. 680), fractional precipitation of the barium salt gave no indication of the presence of two optical isomers and fractional crystallisation of the brucine salt from 50 % alcohol also failed to give any separation. The solubility of the salt in 50 % alcohol, however, was such that crystals of the brucine salt could only be obtained from very concentrated solutions. The crystalline brucine salt could, however, be readily obtained from alcohol-acetone solution.

The specific rotation of the brucine salt (conc. 1.45 %) $[\alpha]_{\text{H}_2\text{O}}^{19} - 31.7^\circ$			
Calculated for $(\text{C}_6\text{H}_{10}\text{O}_4(\text{PO}_4\text{H}_2)(\text{OCH}_3)(\text{C}_{23}\text{H}_{28}\text{N}_2\text{O}_4)_2$	P		2.91 %
Found	P		3.06 %

The brucine salt was converted to barium salt as has been described for the  $\alpha$ - and  $\beta$ -methylhexosidediphosphates.

*Analysis.*

	$\text{OCH}_3$	P	Ba
Found	7.48 %	7.50 %	35.12 %
Calculated for $(\text{C}_6\text{H}_{10}\text{O}_4(\text{OCH}_3)(\text{PO}_4\text{Ba}))$	7.57 %	7.57 %	33.52 %
The specific rotation: $[\alpha]_{\text{H}_2\text{O}}^{19.5^\circ} + 0.92$ (conc. 1.08 %)			

*The hydrolysis of  $\alpha$ - and  $\beta$ -methylhexosidediphosphate with acid.*

Considerable difficulty has been encountered in attempting to remove the methyl group from each of these hexosides while leaving both phosphoric acid groups intact. With complete removal of the methyl group by acid hydrolysis there always occurred a slight removal of the phosphoric acid groups. Under these conditions too rigid a value cannot be placed on the determination of reducing power, as a means of distinguishing the end-point of the elimination of the methyl group. The barium salt of the methylhexosidediphosphoric acid was dissolved in normal hydrochloric acid and kept at  $25^\circ$  until the solution possessed a reducing power which agreed with the complete removal of the methyl group. The optical rotation of the solution was measured at intervals during the hydrolysis, details of which are shown in Tables II and III, and Fig. 2.

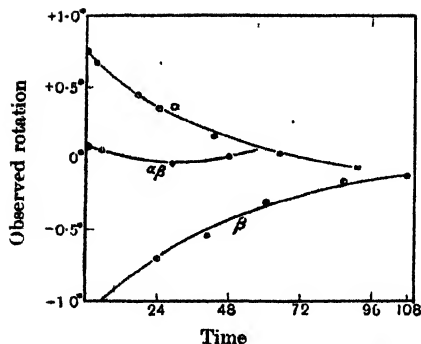


Fig. 2. Optical changes during hydrolysis of  $\alpha$ -,  $\beta$ - and  $\alpha\beta$ -methylhexosidediphosphate with  $N$  HCl at  $50^\circ$ .

Table II.

Approximately 0.30 g. of barium  $\beta$ -methylhexosidediphosphate dissolved in 10 cc. of  $N$  HCl. Temperature  $25^\circ$ . Rotation for mercury green line.

Time (in hours)	Observed rotation	Total P mg.	Inorganic P mg.	Reducing power as glucose mg.
15 mins.	-1.07°	35	Nil	0.28
2 hours	-1.02	34	—	—
3.5 "	-1.00	—	Nil	0.80
24 "	-0.70	—	Nil	12.1
40 "	-0.54	—	0.5	16.8
62 "	-0.30	—	2.1	27.2
86 "	-0.19	—	2.8	33.0
110 "	-0.15	35	3.4	39.0
134 "	-0.08	—	—	—

Table III.

Approximately 0.35 g. of barium  $\alpha$ -methylhexosidediphosphate dissolved in 10 cc. of  $N$  HCl. Temperature  $25^\circ$ . Rotation for mercury green line.

Time (in hours)	Observed rotation	Total P mg.	Inorganic P mg.	Reducing power as glucose mg.
15 mins.	+0.78°	34.3	Nil	0.19
3 hours	+0.67	—	—	—
18 "	+0.57	—	—	13.0
24 "	+0.36	35.0	0.5	14.9
42 "	+0.14	—	—	21.5
66 "	+0.01	35.0	1.4	29.2
90 "	-0.08	—	—	41.3

Both  $\alpha$ - and  $\beta$ -hexosidediphosphoric acids are produced as a result of the removal of the methyl group but as both forms are unstable in solution, the observed rotation in the case of the  $\alpha$ -acid falls while that of the  $\beta$ -acid rises until both values are approximately equal and should, in absence of any hydrolysis of phosphoric acid groups, be equal to the rotation of the commonly known hexosediphosphoric acid in which mutarotation is complete. It will be seen from Tables II and III and Fig. 2, that the final optical value is slightly laevo-rotatory. Presumably this can be explained as due to the production of a small quantity of a laevo-rotatory decomposition product since in both cases a slight production of inorganic phosphate had taken place.

In order to determine the relative rates of hydrolysis of the  $\alpha$ - and  $\beta$ -methylhexosidediphosphates, equal quantities of these two substances were

dissolved in the same volume of  $N/10$  hydrochloric acid and kept at  $50^\circ$  until the reducing power of each corresponded to complete removal of the methyl group. Estimation of the inorganic phosphate at the end of the experiment showed that not more than 2% of the total organic phosphate had been hydrolysed. The relative rates of hydrolysis of these two compounds are shown in Fig. 3.

*Action of emulsin on the  $\alpha$ - and  $\beta$ -methylhexosidediphosphates.*

$\beta$ -Methylfructoside is not hydrolysed by emulsin although this enzyme is generally considered specific in its action on all  $\beta$ -hexosides. Whether emulsin attacks  $\beta$ -methylhexosidediphosphate will, therefore, probably depend on whether or not fructose is the carbohydrate part of the molecule.

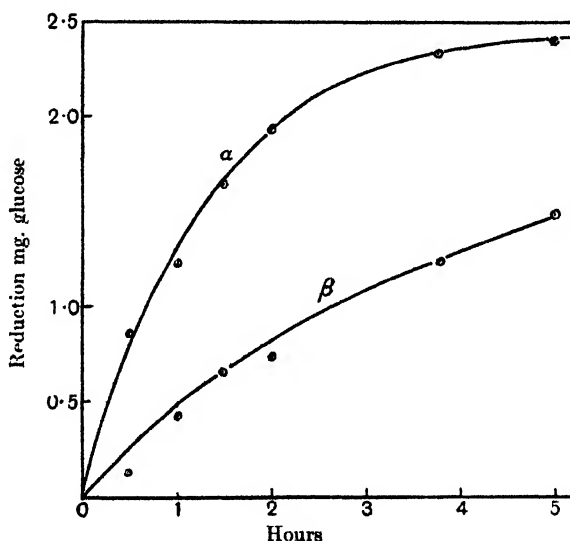


Fig. 3. Hydrolysis of  $\alpha$ - and  $\beta$ -methylhexosidediphosphate by  $N/10$  HCl at  $50^\circ$ .

Two solutions containing 50 mg. of each of the hexosides in 10 cc. water were treated with 0.2 cc. of concentrated emulsin extract prepared from sweet almonds. The solutions were incubated at  $37$ – $38^\circ$  in the presence of toluène. As a control on the activity of the enzyme preparation, solutions of  $\alpha$ -methylglucoside and salicin were treated, under the same conditions, with an equal quantity of enzyme solution. The results are shown in Table IV.

Table IV.

	Reducing power of the solution (mg. glucose) Time in hours					Rotation in 1 dm. tube Time in hours			
	0	3	16	40	64	0	16	40	88
$\alpha$ -Methylhexoside-diphosphate	0.50	0.50	0.48	0.44	0.50	+0.05°	+0.04°	+0.04°	+0.02°
$\beta$ -Methylglucoside	0.38	0.41	0.38	0.38	0.38	-0.06	-0.07	-0.07	-0.09
$\alpha$ -Methylglucoside	0.62	0.62	0.60	0.55	0.63	—	—	—	—
Salicin	1.36	4.11	14.30	17.20	—	-0.39	-0.24	-0.19	-0.19
Boiled enzyme	0.36	0.36	0.31	—	—	-0.01	-0.01	-0.01	—

Emulsin has, therefore, no hydrolytic action on the methoxyl group of the  $\alpha$ - and  $\beta$ -methylhexosidediphosphates. There is, however, a considerable hydrolysis of organic phosphate since the inorganic phosphate present at the end of the experiment was 5.2 % and 7.2 % of the total organic phosphorus for the  $\alpha$ - and  $\beta$ -hexosides respectively. That the liberation of this quantity of phosphorus did not in itself rapidly increase the reducing power of the solution showed that the methyl groups remained attached to the reducing groups of the hexosides.

*Action of invertase on the  $\alpha$ - and  $\beta$ -methylhexosidediphosphates.*

50 mg. of each of the hexosides were dissolved in 10 cc. water and treated with 0.5 cc. of invertase solution. The activity of the enzyme was followed by using control solutions of sucrose,  $\alpha$ -methylglucoside and salicin. The solutions were kept at 37° throughout the experiment. Complete hydrolysis of the  $\alpha$ - or  $\beta$ -compound would yield a reducing solution equivalent to 5.0 mg. glucose. The results of the experiment are shown in Table V.

Table V.

Reducing power of solutions (as glucose, mg.)  
Time in hours

Substance	0	4	20	44	68
$\alpha$ -Methylhexosidediphosphate	1.22	—	2.64	2.64	2.64
$\beta$ -	1.25	1.28	1.58	1.59	1.59
$\alpha$ -Methylglucoside	1.25	1.30	1.30	1.62	1.58
Salicin	2.30	2.28	2.34	2.34	2.64
Sucrose	1.27	32.1	58.0	59.0	59.0
Boiled enzyme	1.20	1.30	1.25	1.39	1.39

There has apparently been some hydrolysis in the case of the  $\alpha$ -hexoside (25 %). In another experiment 32 % of the  $\alpha$ -hexoside was hydrolysed, thus indicating that invertase slowly attacks the  $\alpha$ -, but not the  $\beta$ -compound. Removal of the phosphate groups also occurred to the extent of 10 and 13 % of the total organic phosphorus in the  $\alpha$ - and  $\beta$ -hexoside respectively. Harding [1912], using a crude invertase preparation, found that the phosphoric acid groups of hexosediphosphate were rapidly removed by hydrolysis.

The hydrolytic removal of the phosphate groups in the experiments described above was no doubt due to hexosephosphatase contained in the invertase, which had been prepared from autolysed yeast.

*The methylation of hexosediphosphoric acid.*

As has been described in the introduction, a few preliminary experiments were made, during the earlier part of this investigation, on the methylation of the free hexosediphosphoric acid in methyl alcohol solution.

Barium hexosediphosphate (20 g.) in the form of a fine powder was slowly added to 50 cc. ice-cold methyl alcohol containing sufficient sulphuric acid to precipitate the whole of the barium as barium sulphate. After standing for

a few hours in the cold room ( $0^{\circ}$ ) the whole of the barium sulphate was precipitated. The clear methyl alcohol solution of the hexosediphosphoric acid was then concentrated to a volume of about 10–15 cc. and methylated according to the method of Purdie and Irvine [1903]. The methylation of the compound was carried out in three stages and a large excess of the methylating agents was used. The methyl alcohol solution was mixed with 12 mols. of methyl iodide and warmed on a water-bath. Silver oxide (6 mols.) was then slowly added over a period of 5 hours and the heating continued for some hours after the last addition of silver oxide. The product was then filtered and the residue of silver oxide and silver iodide extracted with boiling methyl alcohol. The filtrate together with the methyl alcohol extracts was evaporated in a desiccator, when a pale yellow syrup was obtained: yield 2 g. This syrup was again methylated using the minimum quantity of methyl alcohol possible to effect solution (about 5 cc.). A non-reducing colourless syrup was obtained which was free from inorganic phosphate but contained 14.5 % of organically combined phosphorus ( $7\text{OCH}_3$  requires 14.1 % P). Estimation of methoxyl groups showed that the substance contained 42.5 % as against 49.5 %  $\text{OCH}_3$  for the fully methylated hexamethyl-methylhexosidediphosphoric acid. Further methylation produced a small quantity of a colourless syrup which contained 47.8 %  $\text{OCH}_3$ , thus indicating a mixture of compounds containing 6 and 7  $\text{OCH}_3$  groups. An attempt to distil this syrup under 1 mm. pressure only gave rise to decomposition. Further work on the production of methylated derivatives of hexosediphosphoric acid is in progress.

*The hydrolysis of  $\beta$ -methylhexosidediphosphate with bone enzyme.*

The discovery by Robison [1923] of an enzyme in ossifying cartilage, which will rapidly remove the phosphoric acid groups of hexosediphosphate by hydrolysis, suggests a further method of attacking the constitution of the hexose portion of the hexosediphosphoric acid molecule. A method of hydrolysing barium hexosediphosphate with the enzyme, the optimum  $p_{\text{H}}$  of which is well on the alkaline side of neutrality, is discussed in a recent paper by Martland and Robison [1927] who point out the difficulty of identifying the liberated hexose after it has remained in alkaline solution for several days. The application of this method to  $\beta$ -methylhexosidediphosphate has given more promising results, as the liberated  $\beta$ -methylhexoside does not undergo the Lobry de Bruyn transformation owing to the substituent methyl group attached to the reducing group.

An experiment using 0.3 g. of barium  $\beta$ -methylhexosidediphosphate after treatment with bone enzyme gave, on concentrating the aqueous solution, a syrup which contained 9.9 %  $\text{OCH}_3$  ( $\beta$ -methylfructoside requires 15.9 %). A solution of the syrup was non-reducing and strongly laevo-rotatory,  $[\alpha]_{\text{H}_2\text{O}}^{18} - 133^{\circ}$ . After boiling a small quantity of the syrup with dilute hydrochloric acid there was a considerable increase in reduction. Control experiments with solutions containing the enzyme together with phosphate

were made and correction made for any optical activity or reducing power due to the enzyme. The few results so far obtained support the evidence of Young and Neuberg that fructose is the hexose part of the hexosediphosphoric acid. Further work on this subject is in progress.

#### SUMMARY.

1. Two isomeric methylhexosidediphosphoric acids have been prepared from yeast hexosediphosphoric acid.

2. A method is described for the separation of these new compounds.

3. The  $\alpha$ -methylhexosidediphosphoric acid is dextro-rotatory

$$[\alpha]_{\text{H}_2\text{O green}}^{20^\circ} + 19.0^\circ,$$

while the isomeric  $\beta$ -compound is strongly laevo-rotatory

$$[\alpha]_{\text{H}_2\text{O green}}^{18^\circ} - 23.0^\circ.$$

4. The barium and brucine salts of both of these compounds are described.

5. The methyl group in  $\alpha$ -methylhexosidediphosphoric acid is more rapidly removed by hydrolysis than that in the corresponding  $\beta$ -compound.

6. Neither compound is hydrolysed by emulsin.

7. Invertase causes a partial removal of the methyl group from the  $\alpha$ -hexoside. The  $\beta$ -methylhexosidediphosphoric acid is not attacked.

8. The methylation of hexosediphosphoric acid yielded a non-reducing syrup consisting largely of the hexamethyl-methylhexosidediphosphoric acid.

9. Bone enzyme rapidly removes the phosphoric acid groups from  $\beta$ -methylhexosidediphosphate, at the same time producing a strongly laevo-rotatory non-reducing substance possessing the properties of a fructoside.

10. A methylhexosidemonophosphoric acid, which was produced in small quantities during the methylation of hexosediphosphoric acid by Fischer's method, has been isolated in the form of its barium salt.

The author wishes to express his most sincere thanks to Prof. A. Harden, F.R.S., and Dr R. Robison for their constant advice and criticism throughout the whole of this investigation.

#### REFERENCES.

- Fischer (1895). *Ber. deutsch. chem. Ges.* **28**, 1160.  
 Harding (1912). *Proc. Roy. Soc. Lond. B.* **85**, 418.  
 Howarth and Hirst (1926). *J. Chem. Soc.* 1858.  
 Irvine and Patterson (1922). *J. Chem. Soc.* **121**, 2696.  
 Lebedev (1909). *Biochem. Z.* **20**, 114.  
 — (1910). *Biochem. Z.* **28**, 213.  
 — (1911). *Ber. deutsch. chem. Ges.* **44**, 2932.  
 Martland and Robison (1927). *Biochem. J.* **21**, 665.  
 Menzies (1922). *J. Chem. Soc.* **122**, 2245.  
 Neuberg and Kretschmer (1911). *Biochem. Z.* **36**, 4.  
 Purdie and Irvine (1903). *J. Chem. Soc.* **83**, 1021.  
 Purdie and Paul (1907). *J. Chem. Soc.* **91**, 289.  
 Robison (1923). *Biochem. J.* **17**, 286.  
 Young (1909). *Proc. Roy. Soc. Lond. B.* **81**, 528.  
 — (1911). *Biochem. Z.* **32**, 178.

## XCI. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. V.

By SYLVESTER SOLOMON ZILVA.

*From the Biochemical Department, Lister Institute, London.*

*(Received May 3rd, 1927.)*

It was previously pointed out [Zilva, 1924] that even the purest antiscorbutic fractions possess the property of reducing ammoniacal silver nitrate in the cold and of decolorising potassium permanganate. In view of the fact that the antiscorbutic activity of solutions is destroyed by exposure to oxygen, it was not unreasonable to assume the existence of a possible connection between the two phenomena. The first obvious problem to solve was, therefore, whether the reduction of either or both of the above reagents was brought about by a substance or a grouping in a substance which was also responsible for the antiscorbutic potency. The experiments of Connell and Zilva [1924] pointed to the fact that this was not the case. In that investigation it was shown that although the conditions conducing to the preservation of the vitamin also conduced to the preservation of the reducing properties of an active solution, the destruction of the antiscorbutic activity and of the reducing properties proceeded at different rates.

Owing to the fact that antiscorbutic solutions lose their activity in the presence of atmospheric oxygen it is customary to imply that the destruction of the physiological activity is due to the direct oxidation of the vitamin. Although there is no experimental evidence against this conception one is not justified in excluding the possibility that the deleterious action of the oxidation process may be at least partly indirect, since the purest fractions so far obtained are impure in the chemical sense. In fact, the stability of the active principle may be conditioned by the presence of some of these accompanying "impurities." With the object of throwing some light on this subject the writer has been endeavouring, during the last few years, to modify active solutions in such a way as to destroy their capacity for reducing ammoniacal silver nitrate and at the same time leave the antiscorbutic activity mainly unimpaired. These efforts have not proved successful. Information, however, having a bearing on this problem has, in the meantime, been forthcoming from a different direction.

Phenolindophenol was found to be rapidly reduced in the air to its leuco-base by decitrated lemon juice and by active fractions derived from that



source. By using this indicator it is, therefore, possible to estimate the reducing capacity of such preparations titrimetrically. Substances with equal or higher oxidation intensities than phenolindophenol, if present, would, of course, not be accounted for by this method. In this empirical way it is nevertheless possible to obtain definite and consistent data of the reducing capacities of antiscorbutically active fractions under various conditions. An opportunity is thus afforded for correlating the two phenomena.

In this investigation it is proposed to show that the reducing agency (the word agency is used for convenience of expression without implying that a single substance is under discussion), although closely associated with the antiscorbutic factor, is not identical with it. There are, however, indications that its presence, most probably amongst other substances, in active solutions, may contribute to the stability of the antiscorbutic potency.

#### EXPERIMENTAL.

It was found convenient to employ, in the experiments to be described, a 0.02 % aqueous phenolindophenol solution. This indicator is red in acid and blue in alkaline solution. All titrations were carried out at  $p_H$  7, since acid solutions decolorise the indicator independently of the reducing properties of the solution, whilst in alkaline solutions, as will be shown later, the reducing agency of the antiscorbutic solution deteriorates. The deleterious action of acidity can be demonstrated by adding a little of the indicator to distilled water adjusted to  $p_H$  4.5 and  $p_H$  3.4. After a few hours the colour of the solutions disappears. In the former case the addition of a few drops of ammonia regenerates the colour, in the latter case the colour is irretrievably lost.

Experiments were at first instituted with the object of studying the behaviour of the reducing agency under those conditions under which the behaviour of the antiscorbutic factor has already been established.

#### *The influence of $p_H$ on the reducing agency.*

Three batches of decitrated lemon juice were adjusted to  $p_H$  5.6, 7 and 9 respectively. Each of the solutions was titrated with the indicator immediately after adjusting the reaction, after 5 hours, and after 22 hours, no precautions having been taken to exclude air. It will be seen from Table I that in the case of the neutral and acid solutions there was comparatively little deterioration of the reducing agency in 22 hours. On the other hand, when the solution was made alkaline almost half of the reducing power was destroyed during the time taken for the adjustment of the reaction (the same batch of decitrated juice was used for the acid experiment). After 2 hours the reducing power was only about one-eighth of that of the original juice and after 22 hours the solution did not reduce the indicator at all. The reducing agency deteriorates, therefore, very quickly in alkaline solution in the presence of air. In this

respect its behaviour is similar to that of the antiscorbutic factor which is very unstable under such conditions [Zilva, 1923].

Table I.

cc. of indicator per 5 cc. of solution

$p_H$	At once	After 5 hours	After 22 hours
5.6	17.1	15	13.7
7	16.5	—	12.0
9	9.4	2	Nil

*Influence of aeration on reducing agency.*

Decitrated lemon juice which had not been previously precipitated with alcohol was adjusted to  $p_H$  7 and air was aspirated through the solution at room temperature. Samples were removed at various intervals for titration purposes. The original preparation required 18 cc. of the indicator for 5 cc. of solution. After  $2\frac{1}{4}$  hours the titre fell to 7 cc.; after 4 hours to 4.9 cc.; after 8 hours to 1.2 cc. No reduction of the indicator could be established after 17 hours. In this case, also, the behaviour of the reducing agency resembles that of the antiscorbutic factor [Zilva, 1922; Daubney and Zilva, 1926]. The decitrated juice at  $p_H$  7.4 aerated for 15 hours was shown to become totally inactivated when tested on guinea-pigs.

*Behaviour of the reducing agency in the process of fractionation.*

In view of the information available concerning the behaviour of the antiscorbutic factor towards lead acetate as a precipitating reagent, it was of interest to follow the distribution of the reducing agency of decitrated lemon juice in the various fractions brought down by this reagent at different hydrogen ion concentrations. The best part of the vitamin is precipitated by lead acetate within the range of  $p_H$  5.4–7.2. Traces, only, of the factor can be precipitated on the alkaline side of this range, whilst on the acid side, no demonstrable quantities are precipitated at all [Zilva, 1927]. The original decitrated lemon juice, the fraction precipitated by neutral lead acetate at  $p_H$  5.4, and the fraction precipitated at  $p_H$  5.4–7.2 after removal of the first ( $p_H$  5.4) fraction from the solution, were titrated daily during a biological test for a period of about 6 weeks. Table II gives the cc. of indicator taken by equivalents of 5 cc. of each preparation in six representative cases chosen at random. It would appear, firstly, that only a part of the reducing agency is accounted for in the precipitates, secondly, that only about 30–50 % of it is present in the fraction which was shown to contain almost the entire antiscorbutic factor of the original juice ( $p_H$  5.4–7.2), and thirdly, that the totally inactive fraction precipitated by lead acetate at  $p_H$  5.4 shows even a higher reducing capacity than the active fraction. It is, therefore, obvious that the reducing power can have no *direct* connection with the antiscorbutic activity. This conclusion was strengthened by further evidence to be dealt with in a later section.

Table II.

Original decitrated juice	Fraction precipitated at $p_H$ 5.4	Fraction precipitated at $p_H$ 5.4-7.2
21	9.8	5.2
18.7	7.1	4.8
11.1	4.4	4.7
12.5	6.0	4.0
15	6.8	5.0
16.5	5	4.5

*Fate of reducing agency in presence of quantities of indicator sufficient to oxidise it only partly.*

When phenolindophenol is reduced and the leuco-base allowed to remain in the air, the latter becomes gradually oxidised. It was, therefore, to be assumed that if a quantity of indicator insufficient to oxidise the reducing agency were added, the reduced compound might possibly become oxidised in the air and eventually reduced again. This process would continue until the reducing agency became totally oxidised. In other words, the presence of a small quantity of the indicator could accelerate the oxidation of the reducing agency. This was actually found to be the case. To each of 5 batches of 5 cc. of decitrated lemon juice (not precipitated with alcohol) were added 6 cc. of indicator. 5 cc. of original juice required 13.6 cc. of the indicator and consequently a further 7.6 cc. was required to neutralise the reducing agency in each flask. The solutions, after standing in the air, were titrated at definite intervals. The first was titrated after 60 minutes and required 4.2 cc. of indicator; the second 3 cc. after 90 minutes; the third 2.3 cc. after 100 minutes; the fourth 1.5 cc. after 110 minutes; the fifth 0.6 cc. after 120 minutes. Soon afterwards, a solution treated as above became coloured, *i.e.* the re-oxidised leuco-compound was not again reduced owing to the disappearance of the reducing agency.

*Behaviour of the antiscorbutic factor in the absence of the reducing agency.*

It is seen from the foregoing experiments that it is possible to remove the reducing agency from antiscorbutic solutions by the addition of phenolindophenol. As this treatment is not drastic there was a likelihood that the antiscorbutic activity would not be destroyed at the same time, especially as it was seen that there is probably no direct connection between the two phenomena. This was actually demonstrated by the following two series of biological tests. In one case the indicator was added to lemon juice, decitrated without the alcohol treatment and adjusted to  $p_H$  7, until it was no longer reduced. In the other case about 14 mg. of phenolindophenol were added per 30 cc. of the decitrated juice. This addition was calculated to oxidise about three-fourths of the reducing agency. Both preparations were then fed to the test guinea-pigs *with the least possible delay*. In both tests, doses of 1.5 cc., 3 cc. and 5 cc. with three animals (250-300 g.) on each dose were employed. Of the series receiving the totally oxidised juice, the three animals

receiving the 1.5 cc. dose died within 43, 49 and 57 days respectively, showing signs of scurvy *post mortem*. All the animals on 3 cc. and 5 cc. doses survived the 60 days of the test period and after being chloroformed were found to be normal at the autopsy. Of the animals receiving the three-fourths oxidised decitrated juice, all, with the exception of one which died of pneumonia after 57 days, survived the 60 days of the test period; only the animals on the 1.5 cc. and 3 cc. doses showed some signs of scurvy at the autopsy. The loss in the antiscorbutic activity in both cases was, therefore, not at all proportionate to the destruction of the reducing agency. These experiments, therefore, prove definitely that there is no direct connection between the two phenomena, and that the antiscorbutic activity persists at least for a short time after the total destruction of the reducing agency in the medium. Attention was consequently directed to the possible function of the reducing agency as a stabilising agency for the antiscorbutic factor and the relative behaviour of the two principles on storage and on heating was studied with this end in view.

*The behaviour of the antiscorbutic factor and the reducing agency on storage.*

Two sources were employed in this experiment, namely ordinary decitrated lemon juice obtained by removing the acids alone in the way already described in previous communications and alcohol-decitrated juice which was further purified by precipitation with alcohol after decitration and concentration. After the removal of the alcohol in the latter case, the decitrated lemon juice was made up to its original volume with a phosphate buffer solution  $p_H$  6.9. The estimations of the reducing agency and the antiscorbutic potency were carried out in both cases in the original preparation, after 24 hours' storage, and after a week's storage. The storage took place in the cold room, the reaction being kept all the time as nearly as possible neutral. The phenolindophenol titrations took place daily shortly before the administration of the doses to the guinea-pigs during the entire period of testing. As one would expect, there was a certain amount of variation in the titres of corresponding preparations from day to day. Tables III and IV give the maximum, minimum and mean titres for 5 cc. of each of the preparations and a summary of the parallel biological tests. It is to be pointed out that although the differences between the maximum and the minimum titrations were great, most of the figures varied more or less in the neighbourhood of the mean.

The two tests can only be compared in a rough way since the antiscorbutic potency is assessed with much less precision by the biological method than the estimation of the reducing agency by titration with phenolindophenol. The results, however, show that the deterioration of the two principles on storage is more or less of the same order, possibly the loss is greater in the case of the reducing agency. This suggests a possible dependence of the antiscorbutic factor on the reducing agency. Further experiments pointed in the same direction.

Table III.

*Ordinary decitrated juice.*

	cc. indicator per 5 cc. of solution		
	Original cc.	After 24 hours' storage cc.	After 8 days' storage cc.
Mean	14.4	11.2	9
Maximum	19.9	18.6	15.8
Minimum	11.3	6.5	1.8

*Alcohol-decitrated juice.*

Mean	11.5	8.8	5.5
Maximum	17.2	13.0	9.2
Minimum	6.5	5.5	2.8

Table IV.

Description of preparation	Dose cc.	Days alive	Remarks	Autopsy
<i>Ordinary decitrated :</i>				
Original	1.5	60	Chloroformed	Slight scurvy
"	1.5	60	"	Normal
"	1.5	60	"	"
After 24 hours' storage	1.5	60	"	Slight scurvy
"	1.5	60	"	"
"	1.5	60	"	Normal
"	3	60	"	"
"	3	60	"	"
"	3	60	"	"
After 8 days' storage	1.5	58	Died	No scurvy
"	1.5	60	Chloroformed	Slight scurvy
"	1.5	60	"	Normal
"	3	60	"	"
"	3	60	"	"
"	3	60	"	"
<i>Alcohol decitrated :</i>				
Original	1.5	58	Chloroformed	Slight scurvy
"	1.5	58	"	Normal
"	1.5	58	"	Slight scurvy
"	3	58	"	Normal
"	3	58	"	"
"	3	58	"	"
After 24 hours' storage	1.5	38	Died	Scurvy
"	1.5	58	Chloroformed	"
"	1.5	53	Died	"
"	3	58	Chloroformed	Normal
"	3	45	Died	Intercurrent disease no scurvy
"	3	58	Chloroformed	Normal
"	5	58	"	"
"	5	58	"	"
"	5	58	"	"
After 7 days' storage	1.5	40	Died	Scurvy
"	1.5	33	"	"
"	1.5	38	"	"
"	3	58	Chloroformed	Slight scurvy
"	3	58	"	"
"	3	38	Died	"
"	5	58	Chloroformed	"
"	5	37	Died	"
"	5	58	Chloroformed	Normal

*Influence of heat on the antiscorbutic factor in the absence of air.*

In connection with another enquiry now in progress it was necessary to ascertain whether heating antiscorbutic solutions at high temperatures in the strict absence of air destroys their potency to any considerable extent. For this purpose ordinary decitrated lemon juice adjusted to  $p_{\text{H}}$  7 was exhausted in an ampoule under a vacuum pump and washed out with nitrogen which was previously shown by absorption with alkaline pyrogallol to be free from appreciable traces of oxygen. This process was repeated three times and the evacuated ampoule, containing the antiscorbutic solution, was then heated for 1 hour in a steam autoclave under a pressure of one atmosphere and cooled before letting in the air. Such preparations made daily (except during the week-end) were tested for their potency in 1.5 cc., 3 cc. and 5 cc. doses, three guinea-pigs being used on each dose. The loss in the antiscorbutic potency was found to be very small since two of the animals on the lowest dose survived the test period of 58 days; the other one died of an intercurrent disease. All these animals, however, showed signs of scurvy at the autopsy after being chloroformed. The guinea-pigs on the higher doses all survived the test period and only in a few cases very slight indications of scurvy were found *post mortem*. Acidified decitrated lemon juice treated as above and tested out in precisely the same way showed a somewhat smaller loss in the antiscorbutic potency. The control animals receiving the untreated decitrated lemon juice were, of course, fully protected on all the doses. I am indebted to Miss S. M. L. Snelus, F.I.C. for assisting me in the preparation and the testing of these juices.

*The behaviour of the antiscorbutic factor and the reducing agency on storage after being heated under anaerobic conditions.*

Decitrated lemon juice treated as described in the preceding section showed very little change in its reducing capacity when titrated with phenolindophenol. Experiments with such preparations after storage yielded interesting results. When crude decitrated lemon juice is stored in the cold room for a week there is comparatively little deterioration either in its antiscorbutic activity or in its reducing capacity. Even the minimum daily dose of 1.5 cc. is practically capable of protecting a young guinea-pig from scurvy for 60 days. In the case of the reducing agency there is a loss of about 30 % during this period. When, however, crude decitrated lemon juice at  $p_{\text{H}}$  7 is heated anaerobically under the conditions described above and then stored for a week the loss in both principles is almost complete. Thus three guinea-pigs on a daily dose of 1.5 cc. of the stored preparation succumbed to scurvy within 4 weeks; two animals on a 3 cc. dose behaved similarly, and two animals on a 5 cc. dose had to be chloroformed after 38 days, scurvy being established at the autopsy. The control guinea-pigs receiving the same autoclaved preparation before storing were alive on all the doses after 38 days. The reducing agency

also disappeared almost entirely during the period of storage. A second experiment confirmed the above observation. These results afford one more illustration of the parallel behaviour of the reducing agency and the antiscorbutic factor.

#### CONCLUSION.

One of the main results which emerges from this investigation is that the reducing capacity of antiscorbutic solutions from lemon juice as measured by the reduction of phenolindophenol is not directly associated with the activity. This is revealed by the facts, first, that inactive fractions derived by precipitating decitrated lemon juice with neutral lead acetate at  $p_H$  5.4 show greater reducing properties than the active fraction derived from the same source, and second, that by fully destroying the reducing capacity by the addition of phenolindophenol in excess, the antiscorbutic activity of decitrated lemon juice remains almost intact, if tested immediately after treatment. In other words, the antiscorbutic factor, if one views it as a definite oxidisable substance, is of a lower reducing intensity than the reducing agency. It is therefore not surprising that any treatment such as aeration, storage, etc., which destroys the antiscorbutic activity also destroys the reducing power of the solution. Does this reducing property of the lemon juice fulfil a protective function in the sense of a "reduction buffer" or is its presence merely accidental? Admittedly the evidence produced in this communication does not offer a conclusive answer. The study of the antiscorbutic solutions from other sources and of the behaviour on storage of active solutions after treatment with phenolindophenol would go a long way towards elucidating this. Preliminary work on the latter problem shows that there is actually a rapid inactivation of the antiscorbutic potency under these circumstances, but as the results are complicated by other factors the final decision must at present be deferred.

No doubt can, however, be entertained about the result obtained in connection with the behaviour of heated decitrated lemon juice on storage. Definite evidence is produced showing that by heating the solution it was modified in such a way as to render it less stable as regards its antiscorbutic potency. The actual character of this modification is still under investigation and it is hoped that information will be forthcoming which will throw more light on the subject. It is significant that in this case also the reducing agency disappears with the deterioration of the antiscorbutic activity. It would almost appear as if the stability of the antiscorbutic factor depends on a chain of reactions which are kept in equilibrium in the living cell and that on damaging the cell the equilibrium is disturbed and the individual links are progressively damaged with the ultimate inactivation of the vitamin. The presence of some impurities would, according to this view, be a necessary condition for establishing the antiscorbutic potency, since the removal of such stabilising substances would inactivate the vitamin before the preparation

could be tested. The author has been faced by many disconcerting results in the chemical purification of the vitamin which could be explained on these lines. This hypothesis leads to another conception. So far the antiscorbutic factor has been studied mainly from the aspect of a principle the presence of which is necessary in the diet of certain animals in order to prevent the derangement of unknown physiological functions eventually leading to scurvy. Has it a function in the plant? Is it present there as a necessary link in the metabolic cycle? The author has shown that by certain manipulations it is possible to purify the antiscorbutic factor in lemon juice to a very great extent, but the issue is gradually being narrowed down and the direct application of chemical methods is becoming less effective. The results of this investigation suggest that the frontal attack on the problem must now be supported by thrusts in other directions.

#### SUMMARY.

(1) Decitrated lemon juice and active fractions derived from that source reduce phenolindophenol to its leuco-compound. In this way the reducing capacity of such solutions can be quantitatively determined.

(2) If insufficient of the indicator to destroy the reducing property of such solutions be added, the reduced compound is re-oxidised in the air and is further reduced by the solution. This alternate reduction and oxidation proceeds until the reducing power of the medium is destroyed.

(3) The reducing agency, like the antiscorbutic factor, is destroyed in alkaline medium in the presence of air, on aerating the active solution and on storage. On fractionating decitrated lemon juice it is, however, found in as high quantities in inactive as in active fractions.

(4) On adding phenolindophenol to decitrated lemon juice until the indicator is no longer reduced and testing the treated solution *immediately*, no very appreciable loss in the antiscorbutic activity is observed.

(5) On heating decitrated lemon juice in a neutral or acid medium in an autoclave at a pressure of one atmosphere for one hour, no very appreciable destruction of the antiscorbutic activity or of the reducing capacity of the solution takes place. On storing, however, both functions deteriorate very much more quickly than in untreated decitrated lemon juice.

(6) It is suggested that the stability of the antiscorbutic factor possibly depends on a chain of reactions, which are kept in equilibrium in the living cell.

The author wishes to express his indebtedness to the Medical Research Council for a personal grant.

#### REFERENCES.

- Connell and Zilva (1924). *Biochem. J.* 18, 632.  
Daubney and Zilva (1926). *Biochem. J.* 20, 519.  
Zilva (1922). *Biochem. J.* 16, 42.  
— (1923). *Biochem. J.* 17, 410.  
— (1924). *Biochem. J.* 18, 632.  
— (1927). *Biochem. J.* 21, 354.



## XCIV. ON THE COMPOSITE NATURE OF THE WATER-SOLUBLE B VITAMIN<sup>1</sup>.

By HARRIETTE CHICK AND MARGARET HONORA ROSCOE.

*From the Department of Experimental Pathology, Lister Institute, London.*

*(Received May 3rd, 1927.)*

IN 1915 McCollum and Davis [1915, 1, 2] came to the conclusion that a water-soluble accessory factor was necessary in diet in addition to the fat-soluble vitamin already discovered by them [1913]. This conclusion was based upon the results of a long series of experiments upon young growing rats. McCollum and his colleagues also expressed the opinion that there existed two, and only two, such accessory factors in diet, which were named respectively water-soluble B and fat-soluble A [McCollum and Kennedy, 1916]. The water-soluble accessory substance was found present in milk, egg-yolk and wheat embryo, was soluble in alcohol as well as in water, and withstood prolonged heating at the boiling point of water.

The distribution and properties of water-soluble B, as far as this was investigated, were similar to those of the so-called antineuritic or antiberberi vitamin which had been discovered by Eijkman [1897] and his colleagues as the result of their studies upon induced polyneuritis in birds and their clinical observations upon the etiology of human beriberi. McCollum and Kennedy [1916] concluded that the water-soluble B vitamin was identical with the antineuritic vitamin and that "in the production of polyneuritis in birds by exclusive rice feeding or exclusive feeding of a ration made up of purified foodstuffs, the degeneration of the nerve cells is the specific result of a lack of water-soluble B" [1916, p. 501]. This opinion was widely adopted and emphasised by other authorities; for example by Drummond [1917] and by the Medical Research Council in their Report upon the Present State of Knowledge Concerning Accessory Food Factors [1919, pp. 35, 40].

There were, however, several facts already recorded which were incompatible with this view. Schaumann [1911], Cooper [1912] and Chick and Hume [1917, 2] had pointed out that some materials, mostly extracts and concentrates made from various natural foodstuffs, which were potent to cure or prevent polyneuritis, were incapable of maintaining the weight of the

<sup>1</sup> In this paper the term *antineuritic vitamin* signifies the substance necessary in a diet to prevent polyneuritis in birds and paralysis in rats. The term *vitamin B* (Goldberger's Pellagra-preventive, P-P, factor) is confined to the water-soluble vitamin needed to prevent the pellagra-like disorder in rats with lesions of the skin and alimentary tract. The term *water-soluble B vitamin*, or *vitamins* (the water-soluble B dietary factor of McCollum and his colleagues) signifies a combination of the above two vitamins, each of which is necessary to maintain growth and health.

experimental birds, whereas others had both actions. Cooper [1912] emphasised this fact. Working with several naturally occurring foods, he showed that the respective daily doses which must be added to the birds' diet of polished rice to maintain body weight and to prevent polyneuritis respectively, were different and that there was no regular relation between them.

During the last ten years, evidence has been steadily accumulating which is in opposition to the theory that these two vitamins are identical, notwithstanding the fact that their distribution is so similar in naturally occurring foodstuffs. A comprehensive review of the evidence on this point was given in a paper by Mitchell [1919]. More evidence has, however, been added since that date, among others by Heller [1923], Willaman and Olsen [1923], Emmet and Stockholm [1920], and Levene and Muhlfeld [1923].

The evidence against the identity of water-soluble B with the antineuritic vitamin may be summarised under three different headings:

1. *Distribution in nature.* While this is generally the same, there are important quantitative differences. Some materials, for example pure wheat embryo, are rich sources of antineuritic vitamin [Chick and Hume, 1917, 1, 2] but poor in water-soluble B [Osborne and Mendel, 1919, 2]. In the case of milk, meat, green leaves and roots and tubers the reverse is true [Cooper, 1914; Hopkins, 1912, 1920; Osborne and Mendel, 1922; Gibson and Concepcion, 1916; McCollum and Kennedy, 1916; Osborne and Mendel, 1919, 1, 2; 1920; 1922; Chick and Hume, 1917, 3; Vedder and Clark, 1912; Sugiura and Benedict, 1918; McCollum, Simmonds and Parsons, 1918.]

Randoin and Lecoq [1926] have shown that certain yeasts of different origin, while possessing equal power to maintain growth, vary considerably in their antineuritic properties.

2. *Heat stability.* Foodstuffs containing these vitamins can be heated to 100° for 1-2 hours without appreciable loss; at higher temperatures, *e.g.* 120°, the antineuritic vitamin has been found much more sensitive than water-soluble B [Chick and Hume, 1917, 2; Sherman and Grose, 1923; Emmet and Luros, 1920].

3. *Solubility and other properties.* Both principles are soluble in water and can be dialysed. Water-soluble vitamin B is less soluble than the antineuritic vitamin in strong alcohol [Funk, 1912; Cooper, 1913; Kinnersley and Peters, 1925; Drummond, 1917], acetone [Abderhalden, 1920; McCollum and Kennedy, 1916; Steenbock, 1917; McCollum and Simmonds, 1918] and benzene [McCollum and Simmonds, 1918; McCollum and Kennedy, 1916]. Both vitamins can be adsorbed upon animal charcoal or fuller's earth, but adsorption appears to be more complete in case of water-soluble B and recovery more difficult after processes involving precipitation [Drummond, 1917].

Further light has been shed upon the problem recently by the investigations of Goldberger and his colleagues upon pellagra. Those workers have been led to abandon their previous theory that the cause of this disorder is to be traced to an inferior biological value of the protein in the diet. In their

attempts to prevent human pellagra they found that while such a valuable protein as caseinogen (up to 90 g. daily) gave disappointing results, liberal daily supplements of butter-milk (1200 g.), fresh meat (200 g.) or 30-50 g. dried brewer's yeast (and less of certain extracts), were able to cure or prevent pellagra in persons receiving diets on which this disease might confidently be expected to develop [Goldberger and Tanner, 1924, 1925; Goldberger, Wheeler, Lillie and Rogers, 1926]. These foods are all rich sources of water-soluble B and after extending the work to a series of observations on young growing rats [Goldberger, Wheeler, Lillie, and Rogers, 1926; Goldberger and Lillie, 1926] the conclusion has been reached that the results can best be explained by supposing that what McCollum and Kennedy described as water-soluble B vitamin consists of two substances. These are (1) a pellagra-preventive, P-P, factor, and (2) an antineuritic vitamin, both constituents being required to maintain growth and health in the young rat, (1) being less soluble in alcohol than (2) and more resistant to heat. The facts on which this theory is based are, shortly, these. When young rats were maintained on a basal diet deprived of the water-soluble B vitamin but including as much as 30-40 % of yeast heated in an autoclave at 15 lb. pressure for 2½ hours, growth was not maintained and death occurred after a period of 8-10 weeks, preceded in some cases by symptoms of paralysis. If, however, the diet was further supplemented by a small daily dose of an alcoholic extract of corn-meal to provide antineuritic vitamin, the animal grew or remained in normal health even if the dose of autoclaved yeast were much diminished. If, on the other hand, the basal diet was supplemented *only with the antineuritic substance*, growth was not maintained, even when a comparatively large dose was administered. No paralysis occurred but, after a variable period following the arrest of growth, a number of such animals developed a series of symptoms which were considered to resemble human pellagra [Goldberger and Lillie, 1926]. The shortest time taken for development was 7 weeks. The symptoms included ophthalmia, loss of fur and characteristic dermatitis on ears, paws and neck. With the inclusion of a small amount, 6 %, of a preparation made with acidified water from autoclaved yeast, these symptoms cleared up and growth was resumed.

The present experiments confirm the work of Goldberger and his colleagues and support the conclusion drawn by those workers and the other observers already referred to, that McCollum's water-soluble B vitamin and Eijkman's antineuritic substance are not identical.

The work arose out of an examination of the alcoholic extract made from the material adsorbed on charcoal in Peters' [1924] process for purification of the antineuritic vitamin contained in brewer's yeast. The extract had been prepared by Sir C. J. Martin in the hope that it might provide a possible standard for comparison in the titration of foodstuffs for content of the water-soluble B vitamin. Prof. Peters had told us that although the material was highly potent for the cure of polyneuritis induced in pigeons on a diet of

polished rice, he had no experience of its capacity to maintain growth in rats on a diet deprived of the water-soluble vitamin B.

This was investigated, and it was found that the extract would not encourage growth. If, however, supplemented with a daily ration of yeast heated at 120° for 5 hours to destroy the antineuritic vitamin, the rats grew normally and health was maintained.

A series of experiments has been carried out in which yeast and wheat embryo have been taken as sources of the two water-soluble B vitamins. Various extracts and products have been prepared from these and used both alone and in combination, to provide supplementary rations for the basal diet deprived of these vitamins. Observation has been made of the different pathological conditions developed in the rats and a quantitative study made of the substances required for prevention and cure.

#### EXPERIMENTAL.

The following materials have been prepared and investigated.

*Yeast. Whole dried yeast.* Fresh brewer's yeast was washed by kneading in ice-cold water to a smooth paste, filtering and pressing, the operation being repeated three or four times. The washed and pressed yeast was dried at 37° for 3 days and ground to a powder in a mill.

*Autoclaved yeast.* The above dried yeast was heated for 5 hours in an autoclave at 120° and then dried again at 37°. It did not alter in weight but the colour changed from light to dark brown.

*Yeast extract* [Peters' method, 1924]. The material used was prepared from the extract with acidified 50 % alcohol (alcohol 50 : water 50 : concentrated HCl 1, by volume) of the material adsorbed on animal charcoal after the precipitations by lead acetate and acid mercuric sulphate had been carried out. In our preparation the traces of mercury remaining were removed, before adsorption with animal charcoal, by passing  $\text{SH}_2$  through the solution. The precipitation was completed and excess of sulphuric acid removed by adding the appropriate amount of  $\text{BaCO}_3$ , excess being avoided. The acid alcoholic extract was concentrated at a low temperature under reduced pressure and the residue taken up in water. 1 cc. of the final solution administered was equivalent to about 7 g. of the original washed yeast (dry weight).

*Wheat embryo.* The wheat embryo was a specially pure specimen containing very little bran; it was dried at 37°.

*Autoclaved wheat embryo* was prepared by autoclaving for 5 hours at 120° and then drying at 37°.

Two extracts were prepared from the wheat embryo.

*Absolute alcohol extract.* Wheat germ dried at 37° was shaken at room temperature for periods of 5 hours with each of two changes of absolute alcohol. The filtrates were taken almost to dryness under reduced pressure at approximately 40° and the residue shaken up with ether and acidified water. The water-soluble fraction, after being dried and redissolved in water, was tested. It is called the *absolute alcohol extract*.

*85 % alcohol extract.* The wheat germ used in the previous extraction was re-extracted by shaking at room temperature for periods of 5 hours with each of two changes of 85 % alcohol (85 cc. absolute alcohol made up to 100 cc. with distilled water). The combined filtrates were reduced to dryness as before and taken up in an amount of water equivalent to the original weight of wheat germ. This is the *85 % alcohol extract*.

The following are the details of the experiments. Black and white or albino rats were used and were placed upon the experimental diet when 3-4 weeks old and weighing 40-50 g. They were kept in cages with screened

wire bottoms of  $\frac{1}{8}$ -inch mesh to prevent contact with the faeces and probable ingestion [see Steenbock, Sell and Nelson, 1923]. The diet was given in a semi-liquid condition in small pots.

The basal diet deprived of water-soluble B vitamins, hereafter called Diet K, consisted of:

Purified caseinogen (specially extracted with ether and alcohol)	21 parts
Rice starch ... ..	63 "
Cotton-seed oil ... ..	11 "
Salt mixture <sup>1</sup> ... ..	5 "

From 0.05 to 0.1 g. cod-liver oil was given to each rat daily according to the size.

1. *Effect on rats of diets lacking in the antineuritic or both water-soluble B vitamins.*

When young rats, 40–50 g. in weight, are placed upon the basal diet K, very slight growth may take place, but the appetite fails and the body weight gradually declines. After 3–4 weeks the body weight is usually about 35 g., the animals are emaciated and present a feeble and miserable appearance. If untreated, they die, usually without any obvious symptoms other than those of inanition.

Table I. *Effect of yeast and wheat embryo—dried, autoclaved or given as extracts—on the cure and prevention of the collapse (so-called "neuritis") occurring in young rats after 3–4 weeks on diet K deprived of water-soluble B vitamins.*

Exp.	Material	Daily dose required for prevention	Daily dose required for cure
A	Yeast, dried	—	0.05 g.
	" autoclaved	None with 0.2 g.	No cure or alleviation with 0.2 g.
	" extract (Peters)	—	0.1 cc.*
B	Wheat embryo, dried	—	0.1 g.
	" autoclaved	None with 0.4 g.	No cure or alleviation with 0.2 g.
	" extract with absolute alcohol	—	No cure or alleviation with equiv. of 1.0 g. wheat germ
	" extract with 85 % alcohol	0.2 cc.†	0.3 cc.

\* Equivalent to 0.7 g. yeast.

† Equivalent to 0.2 g. embryo.

Symptoms of paralysis (see below) were only rarely observed in animals receiving this treatment. Such was also the experience of Drummond, who noted paralytic symptoms in 3 only out of a large number of young rats [Drummond, 1918] on a diet deprived of water-soluble vitamins. Schaumann [1910] observed nervous symptoms in adult rats after 3–4 weeks on a diet of horseflesh heated with alkali to 120° for 1 hour, or of rye bread similarly treated. Hofmeister [1922] has given a detailed study of the paralysis in rats caused by lack of water-soluble vitamins in the diet. He found that the

<sup>1</sup> Salt mixture No. 185 [McCollum, Simmonds and Pitz, 1917].

condition could be best studied when developed in a more chronic form and was of the opinion that when this happened it was probable that the deficiency was not quite complete, otherwise death occurred swiftly and suddenly. In the present experiments with young rats death from inanition usually occurred before there had been time for the development of paralysis. In one series of experiments however (litter 541, Table IV) 5 out of 8 rats in one litter developed symptoms of paralysis within 33 days after receiving the experimental diet. The onset of the paralysis occurred as described by Hofmeister. It was marked by loss of appetite and great restlessness; there was also a progressive loss of co-ordination and of power in the hind legs. The rats swayed from side to side in walking and sometimes would fall down on one side. Later on, the animals maintained a position with the back hunched up and the head bent forward; they became unable to stand upright unless holding on with the forepaws and frequently fell over upon the head. Two animals (rats 29 and 31, Table IV) in this condition were cured with 0.1 cc. of Peters' yeast extract, and three died.

In the majority of cases definite symptoms of paralysis were not observed, and it was at the time of general collapse, occurring after 3-4 weeks upon the experimental diet K, that the effect of curative substances was tested (see Table I). Administration of Peters' antineuritic extract (0.1 cc. daily, equal to 0.7 g. yeast) or the (85 %) alcoholic extract of wheat embryo (0.3 cc. daily, equivalent to 0.3 g. embryo) restored the animal to some degree of health, and weight was maintained for a considerable period, although there was no significant increase. These extracts also cured paralytic symptoms promptly in cases where these were observed (rats 29 and 31, Table IV).

The extract from wheat embryo made with absolute alcohol was without effect in relieving the symptoms of paralysis (see rats 28, 32, 33, Table IV). The antineuritic substance in wheat embryo thus appears to be insoluble or only sparingly soluble in absolute alcohol; that present in the extract prepared from dried yeast by the method of Kinnersley and Peters [1925] is stated however to be soluble in absolute alcohol.

If dried yeast or wheat embryo were given in sufficient amount (yeast 0.05 g.; wheat embryo 0.1 g.) the animals were cured, were restored to health and also began to grow. In the autoclaved yeast or autoclaved wheat germ, on the other hand, the antineuritic properties had been destroyed by the strong heating. When these were administered no cures were effected in rats showing paralysis (rat 28, Table IV) or in those suffering from inanition, and the animals died in the same way as those which were untreated.

## 2. *Development of "pellagra" in rats due to lack of the second of the water-soluble vitamins.*

Young rats which received the basal diet K supplemented with alcoholic extracts made from yeast or wheat embryo did not grow but showed no signs of collapse at 3-4 weeks. After about 6-8 weeks had elapsed they sometimes

died without showing any special external symptoms, but in other cases they gradually developed the condition described by Goldberger and his colleagues (see above) and considered by these workers to resemble human pellagra. The body weight was usually maintained (rat 68, Curve III, Fig. 2), but the animals were in poor condition, they were thin and active, with rough coat and with the

Table II. *Effect of yeast and wheat embryo—dried, autoclaved or given as extracts—on the cure and prevention of “pellagra” occurring in young rats after 6–8 weeks upon diet K, deprived of water-soluble B vitamins, but supplemented by the antineuritic vitamin.*

Exp.	Material	Daily dose required for prevention, g.	Daily dose required for cure, g.
A	Yeast, dried	0.1 + (0.05 –)	—
	„ autoclaved	0.2 + (0.1 –)	0.2
	„ extract (Peters)	None with 0.3 cc.*	—
B	Wheat embryo, dried	0.2	—
	„ autoclaved	0.4	0.4 did not cure
	„ extract with 85 % alcohol	0.8 cc.† delayed onset	—

\* Equivalent to 2.1 g. yeast.

† Equivalent to 0.8 g. embryo.

In cases where no doses are given marked with the – sign, smaller doses were not tried.

urine often slightly blood stained. Loss of hair took place, and red inflamed patches of skin appeared on the nose and on the backs of the fore feet, which later became oedematous. There was a marked conjunctivitis and in some cases the ears also became red and thickened. The inflamed areas spread and often involved the hind legs and whole areas round the nose and mouth; sometimes the chest became bare and inflamed. The animals survived 2–3 weeks or longer in this condition before death occurred. On *post mortem* examination there were no definite macroscopic signs except a very unhealthy condition of the whole alimentary tract, especially in the small intestine which showed signs of inflammation with atrophy of the mucous membrane and often contained blood-stained mucus.

The condition was also developed in rats which had been saved from collapse on basal diet K by administration of the alcoholic extracts of yeast (rat 12, Curve II, Fig. 1) or of wheat embryo (rat 52, Curve I, Fig. 2). The time elapsing was usually 3–4 weeks after the previous collapse and 6–8 weeks from the beginning of the experiment.

This “pellagrous” condition was cured slowly but surely by addition to the diet of a small daily ration (0.2 g. and upwards) of autoclaved yeast (see Table II A and rat 12, Curve II, Fig. 1), and at the same time growth was restored. Several days, however, usually elapsed before there was a definite improvement. The skin lesions gradually healed, desquamation took place on chest and paws and round nose and mouth; thickened, cheesy layers peeled from the ears, leaving them thin and papery. In about 2 weeks the skin was healed and hair was beginning to grow.





0.2-0.4 g. daily of dried yeast or 1.0 g. of wheat embryo (Table III, Exps. A and C).

When the rats received doses of autoclaved yeast up to 0.2 g. daily (rat 62, Curve IV, Fig. 1) there was no evidence of growth, but if supplemented by 0.1 cc. daily of the yeast extract, steady growth took place, although it was subnormal (7 g. a week, Table III B). A normal increase in body weight took place when the rats received 0.4 g. autoclaved yeast together with 0.1 cc. of the yeast extract. Since neither of these materials given alone in similar amount could maintain growth, but could respectively relieve the "pellagrous" and neuritic symptoms, the conclusion is obvious that whole yeast contains both principles, that both are necessary for growth and health, that the antineuritic vitamin is destroyed in the autoclaved yeast and that vitamin B (Goldberger's P-P factor) is absent from this particular yeast extract.

Drummond [1918] found that extracts made from yeast by methods involving precipitation processes had lost the power of promoting growth in rats, whereas a number of other observers, including Peters and ourselves, find similar extracts to be potent antineuritic substances.

Table III. *Effect on growth of young rats of vitamin B, or the "pellagra-preventive" (P-P) factor, in presence of adequate antineuritic vitamin.*

Ex- per- iment	Material	Daily dose, g.	Given as preventive		Given as cure for collapse due to lack of antineuritic vitamin or of vitamin B	
			No. of animals	Average weekly increase in body wt. g.	No. of animals	Average weekly increase in body wt. g.
A	Yeast, dried	0.05	—	—	1	1
		0.1	—	—	1	3
		*0.2	1	11	3	9
		0.3	—	—	1	7.5
		0.4	1	23	—	—
B	Yeast, autoclaved (antineuritic vitamin supplied as Peters' yeast extract)	0.1	—	—	1	4
		0.2	1	7	3	5
		*0.3	—	—	2	13
		0.4	1	10	1	10
C	Wheat embryo, dried	0.1	—	—	1	3
		0.2	1	2	3	3
		0.4	—	—	2	1.5
		0.6	1	6	1	6
		*1.0	2	13	—	—
		1.5	1	12	—	—
D	Wheat embryo, autoclaved (anti- neuritic vitamin supplied as 85% alcoholic extract of wheat em- bryo or as Peters' yeast extract)	0.2	—	—	1	0
		0.4	1	2	2	4
		0.6	—	—	1	4
		0.8	—	—	2	4
		1.0	1	5	—	—
E	Wheat embryo, dried + 0.3 g. autoclaved yeast	0.2	1	15	—	—
		0.4	2	15	—	—
		0.6	1	15	—	—

\* Dose required for normal growth.



*Wheat embryo.* The evidence of the existence of the two water-soluble B vitamins in wheat embryo is not quite as dramatic as with yeast. The explanation seems to be that the wheat embryo, if not mixed with other portions of the grain, is comparatively poor in the P-P constituent, while a rich source of the antineuritic factor. Osborne and Mendel [1919, 2], in growth tests with rats, found that commercial wheat embryo, i.e. a sample admixed with bran, was a better source of water-soluble B vitamin than a very pure sample of the embryo picked off the seed by hand in the laboratory. The sample of embryo used in the present case was specially pure and contained only a very small proportion of bran or other products. With their commercial wheat embryo Osborne and Mendel obtained good growth with doses less than 0.2 g. daily; with the present specimen 1.0 g. daily was required (see Table III C).

That the P-P factor is present in wheat embryo in very small amount compared with its content of antineuritic vitamin is also evident from the following facts.

(1) When the whole wheat embryo was supplemented with a small daily dose, 0.3 g., of autoclaved yeast, normal growth (an increase of 15 g. weekly) was obtained with a daily dose of only 0.2 g. daily of the embryo (Table III E).

(2) Using the autoclaved embryo, supplemented with antineuritic vitamin from another source, only subnormal growth showing 5 g. increase in weight per week was obtained with a daily dose of 1 g. (Table III D).

(3) The 85 % alcoholic extract of the embryo, which should contain both constituents, if present in the original material, was found to be a good source of the antineuritic vitamin (see Table I B) but to have only a slight effect in preventing the onset of "pellagra" (Table II B).

#### DISCUSSION.

From the work of others and of ourselves described above, it is clear that the water-soluble B dietary factor of McCollum and his colleagues is not one substance but two at least. McCollum and Kennedy [1916] were evidently mistaken when they adopted the simplest interpretation of the facts at their disposal and pronounced water-soluble B to be identical with the antineuritic substance concerned with the prevention and cure of polyneuritis in birds.

As there are two vitamins two names are required. We would propose to retain the name *antineuritic vitamin* for the antineuritic substance, discovered by Eijkman in 1897 which cured and prevented polyneuritis in birds fed upon a diet of polished rice and was later found to be necessary to prevent marasmus and paresis in mammals.

We suggest that the name *vitamin B* be provisionally confined to the second water-soluble vitamin contained in the dietary factor B discovered by McCollum and Davis in 1915. The substance provisionally called the pellagra-preventive or P-P dietary factor by Goldberger and his colleagues [1926] appears to be vitamin B in this restricted sense. Its presence in a diet is necessary to maintain health in young rats and to prevent decline and death

accompanied by severe skin lesions. Goldberger and his colleagues consider that this condition is the analogue of human pellagra and suggest that deficiency of this vitamin is the cause of the human disease.

As both these water-soluble vitamins are necessary for continued growth and health, it follows that both are present in substances hitherto found to satisfy the requirements of McCollum's water-soluble B, as originally described.

These two vitamins, while both soluble in water, appear (1) to have different solubilities in strong alcohol, benzene and acetone, (2) to possess differing capacities for adsorption on various finely divided solids and (3) to have a markedly different resistance to heat. These differences have made it possible to separate them from one another as they occur in yeast and in wheat embryo, and have provided a method for the study of one apart from the other. On further investigation it may prove that their distribution in nature is by no means as similar as it now appears to be.

In making the above suggestions with regard to nomenclature, we fully realise the incomplete state of our present knowledge concerning the water-soluble B vitamins and the possibility that McCollum's water-soluble B may eventually be shown to contain more constituents than the two dealt with in this paper. The principles responsible for the effects described in experiments of Hartwell [1922, 1, 2], Drummond and Reader [1926], Boas [1927], may or may not prove to be identical with one or other of these two vitamins already differentiated.

#### SUMMARY.

1. Experiments are described with young growing rats which confirm the views held by many investigators, and recently emphasised by Goldberger and his colleagues, indicating that the water-soluble B vitamin of McCollum and Kennedy contains at least two constituents.

(i) The *antineuritic* (or antiberiberi) vitamin which prevents or cures the collapse, accompanied in some cases with symptoms of paralysis, and leading to death, which occurs in young rats of 40–50 g. weight after 3–4 weeks upon a diet devoid of water-soluble B vitamins. It is identical with the antineuritic substance, discovered by Eijkman in 1897, necessary to prevent polyneuritis in birds fed upon a diet of polished rice.

(ii) The *vitamin B sensu strictu*, identical with the pellagra-preventive or P-P factor of Goldberger and his colleagues. This vitamin can prevent or cure the marasmus accompanied by severe skin lesions, which develops in young rats after 6–8 weeks upon the deficient diet. The effect of its absence can only be demonstrated in cases where the animal is provided with the antineuritic factor in order to prevent the collapse which otherwise would occur at an earlier date.

All known substances containing the water-soluble B vitamin, as defined by McCollum and his colleagues, contain both constituents, since both are necessary for maintenance of growth and health.

2. It is suggested that the term *vitamin B* should be confined to this second water-soluble vitamin (Goldberger's P-P factor), distinct from the antineuritic vitamin, but commonly accompanying it in natural foodstuffs.

3. Yeast is a rich source of both water-soluble B vitamins; pure wheat embryo, while rich in the antineuritic vitamin, was found to be comparatively poor in vitamin B (Goldberger's P-P factor).

4. Vitamin B (P-P factor), as was shown by Goldberger and his colleagues, is much more thermostable than the antineuritic vitamin. A preparation practically free from the antineuritic vitamin can be obtained by heating yeast in an autoclave at 120° C. for 4-5 hours.

5. The strongly antineuritic material extracted from the charcoal in Peters' method of purifying the antineuritic vitamin in yeast was found to be devoid of vitamin B (Goldberger's P-P factor).

We wish to acknowledge our indebtedness to Sir C. J. Martin for suggesting this research to us and also for helping us throughout the course of the work with his constant advice and criticism.

#### REFERENCES.

- Abderhalden (1920). *Pflüger's Arch.* **173**, 260.  
Boas (1927). *Biochem. J.* **21**, 712.  
Chick and Hume (1917, 1). *Proc. Roy. Soc. Lond. B*, **90**, 44.  
—— — (1917, 2). *Proc. Roy. Soc. Lond. B*, **90**, 60.  
—— — (1917, 3). *J. Roy. Army Med. Corps*, **29**, 121.  
Cooper (1912). *J. Hyg.* **12**, 436.  
—— (1913). *Biochem. J.* **7**, 268.  
—— (1914). *J. Hyg.* **14**, 12.  
Drummond (1917). *Biochem. J.* **11**, 255.  
—— (1918). *Biochem. J.* **12**, 25.  
Drummond and Reader (1926). *Biochem. J.* **20**, 1256.  
Emmet and Luros (1920). *J. Biol. Chem.* **43**, 265.  
Emmet and Stockholm (1920). *J. Biol. Chem.* **43**, 286.  
Eijkman (1897). *Virchow's Arch.* **148**, 523.  
Funk (1912). *J. Physiol.* **43**, 395.  
Gibson and Concepcion (1916). *Philippine J. Sci.* **11**, 119.  
Goldberger and Lillie (1926). *U.S.A. Public Health Reports*, **41**, 1025.  
Goldberger and Tanner (1924). *U.S.A. Public Health Reports*, **39**, 87.  
—— — (1925). *U.S.A. Public Health Reports*, **40**, 54.  
Goldberger, Wheeler, Lillie and Rogers (1926). *U.S.A. Public Health Reports*, **41**, 297.  
Hartwell (1922, 1). *Biochem. J.* **16**, 78.  
—— (1922, 2). *Biochem. J.* **16**, 825.  
Heller (1923). *J. Biol. Chem.* **55**, 385.  
Hofmeister (1922). *Biochem. Z.* **128**, 540.  
Hopkins (1912). *J. Physiol.* **44**, 425.  
—— (1920). *Biochem. J.* **14**, 721.  
Kinnersley and Peters (1925). *Biochem. J.* **19**, 820.  
Levene and Muhlfield (1923). *J. Biol. Chem.* **57**, 341.  
McCollum and Davis (1913). *J. Biol. Chem.* **15**, 167.  
—— — (1915, 1). *J. Biol. Chem.* **23**, 181.  
—— — (1915, 2). *J. Biol. Chem.* **23**, 231.  
McCollum and Kennedy (1916). *J. Biol. Chem.* **24**, 491

- McCollum and Simmonds (1918). *J. Biol. Chem.* **33**, 55.  
 McCollum, Simmonds and Parsons (1918). *J. Biol. Chem.* **36**, 197.  
 McCollum, Simmonds and Pitz (1917). *J. Biol. Chem.* **29**, 521.  
 Medical Research Council (1919). *Special Report Series*, No. 38.  
 Mitchell (1919). *J. Biol. Chem.* **40**, 399.  
 Osborne and Mendel (1919, 1). *J. Biol. Chem.* **37**, 187.  
 ——— (1919, 2). *J. Biol. Chem.* **37**, 557.  
 ——— (1920). *J. Biol. Chem.* **41**, 451.  
 ——— (1922). *Biochem. J.* **16**, 363.  
 Peters (1924). *Biochem. J.* **18**, 858.  
 Randoin and Lecoq (1926). *Compt. Rend. Acad. Sci.* **182**, 1564.  
 Schaumann (1910). *Beihefte Arch. Schiff. Trop. Hyg.* **14**.  
 ——— (1911). *Trans. Soc. Trop. Med. Hyg.* **5**, 59.  
 Sherman and Grose (1923). *Proc. Soc. Exp. Biol. Med.* **21**, 11.  
 Steenbock (1917). *J. Biol. Chem.* **29**, Proc. xxvii.  
 Steenbock, Sell and Nelson (1923). *J. Biol. Chem.* **55**, 399.  
 Sugiura and Benedict (1918). *J. Biol. Chem.* **36**, 171.  
 Vedder and Clark (1912). *Philippine J. Sci. Sect. B.* **7**, 423.  
 Willaman and Olsen (1923). *J. Biol. Chem.* **55**, 815.

# XCV. THE EFFECT OF DESICCATION UPON THE NUTRITIVE PROPERTIES OF EGG-WHITE.

BY MARGARET AVERIL BOAS (*Beit Memorial Research Fellow*).

*From the Department of Experimental Pathology, Lister Institute, London.*

(Received May 3rd, 1927.)

IN 1922 I was carrying out some experiments upon the calcium and phosphorus retention of rats, for which it was desirable to use a basal diet containing only small amounts of calcium and phosphorus. Egg-white was suggested as a suitable source of protein. Osborne and Mendel [1913, 1, 2; 1915] had shown that young rats could satisfy their nitrogen requirements from a diet in which ovalbumin formed 12 % of the dry weight of the diet and was the sole source of protein, and Bond [1922] had demonstrated satisfactory growth in young rats fed upon a diet containing dried egg-white at a 20 % level as the only protein. For reasons of economy it was decided to use the same source of egg-white as that employed by Bond; this was a dried undenatured preparation of Chinese origin. It was dissolved in water and coagulated by heat before being incorporated in the rest of the diet, for both Bond and Luce had found that the ingestion of raw egg-white by rats was invariably followed by diarrhoea.

The diet, which will be referred to as the DW diet, had the following composition:

Dried egg-white	100 g.	Lemon juice	25 cc.
Wheaten starch	250 g.	Marmite	25 g.
Normal salt mixture <sup>1</sup>	25 g.	Distilled water	300 cc.
Hardened cotton seed oil	75 g.		

Each rat received in addition 3 to 5 drops of cod-liver oil daily.

It was found, however, that when 3 weeks old rats were fed on this diet they invariably began to lose weight after a period of about 21 days, and developed a particular pathological condition which is described in detail below, and of which the most striking symptoms were dermatitis, baldness and spastic gait leading on to death.

*The symptoms.* The rats are as a rule from 21–24 days old when the experiment is started. They grow well and are usually in good health for from 2 to 3 weeks. Then red scaly patches appear at the corners of the mouth, the coat becomes rough and sticky and the long hairs fall out. The fur on the abdomen shows at first a characteristic ribbed appearance, followed by the development of bald areas. Meanwhile the red patches spread to other parts

<sup>1</sup> McCollum, Simmonds and Pitz [1917].

of the body and the picture is one of an eczematous dermatitis. There are even skin haemorrhages in severe cases. The region round the mouth is always the most severely affected, though there is often such marked blepharitis that the eyes are closed. The loss of hair is often extensive. In a few cases oedema of the feet has been seen but this does not usually occur. These rats always have a distinctive somewhat musty smell, probably due to some constituent of the urine. The body weight remains stationary for a week or two, but falls slowly during the second stage of the disease. This is reached about 2 to 3 weeks after the development of the first signs of deficiency. To the dermatitis, symptoms of nervous upset are now added. There is pronounced spasticity of the limbs, particularly of the hind legs, and the back is arched. The rat assumes in many cases a kangaroo-like posture. The accompanying plate shows the condition at this advanced stage. Some of the rats do not show marked spasticity but assume a crouching attitude and display a curious swimming movement with the front paws. Death, which occurs in the final phase, is preceded by a rapid loss of weight, and the animal shows signs of extreme cyanosis. *Rigor mortis* sets in rapidly. *Post mortem* there is an almost complete absence of fat and the skin is infiltrated and vascularised, but these are the only apparent abnormalities, the organs seeming perfectly normal. No extensive histological study has yet been carried out but sections of liver, kidney, thyroid, spleen and adrenals failed to show any marked changes.

These results were so striking that it appeared that egg-white could not contain proteins satisfactory for complete nutrition and a short note was published [Boas, 1924, 1] to this effect. After this paper appeared, Prof. Mendel wrote suggesting that there must be some important difference between the methods I used and those employed by him, since he had repeatedly found egg-white to be an adequate source of protein for young rats. Shortly after this Dr Helen Mitchell told me that she had used the dried white of hard-boiled eggs with complete success as the sole source of protein for rats [Mitchell, 1925].

*The use of fresh egg-white.* The experiments were therefore repeated using the fresh white of English or imported eggs instead of the Chinese preparation, and the results showed that I had been too hasty in concluding that egg-white was unsatisfactory as a source of protein. The rats fed upon the fresh egg-white diet grew well and remained in good condition (see Fig. 1). A second note was published [Boas, 1924, 2] withdrawing my former conclusions, and attributing the results obtained to some unknown factor peculiar to the Chinese egg-white.

*The question of preservatives.* It seemed probable that this unknown factor in the Chinese egg-white might be some toxic preservative. A very thorough investigation carried out in the Government Laboratory by the courtesy of Sir Robert Robertson, however, failed to reveal the presence of any known preservative.

*The influence of the nature of the starch.* I also communicated with Miss



Bond and learnt that although she had long used the Chinese dried egg-white, yet she had never observed in her rats the symptoms I described. The only qualitative difference between the two diets lay in the nature of the starch, as Bond used potato-starch and I used wheat-starch. This difference did not seem likely to be significant but upon investigation it proved to be of the utmost importance. Rats were fed upon the DW diet until the symptoms were developed to such a degree that death would have been expected in a week or less. They then received a diet similar in all respects to the DW diet except for the presence of potato-starch instead of wheat-starch. There was an immediate increase in weight, new hair appeared and a rapid and lasting cure resulted. Furthermore rats fed on the diet prepared with potato starch from the time of weaning grew well and showed none of the symptoms (Fig. 1). That the wheat-starch was not in itself responsible was clear, for when caseinogen was the protein used the nature of the starch was immaterial.

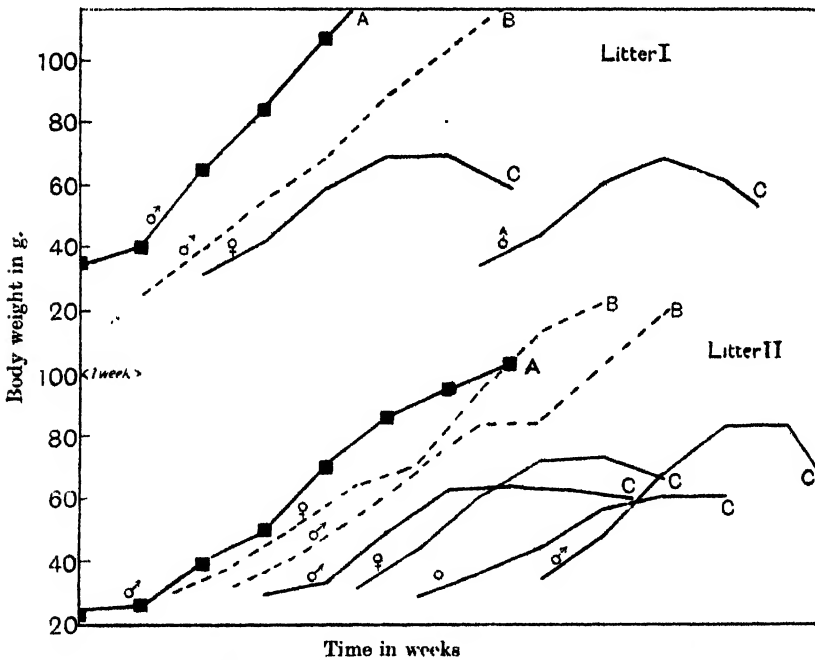


Fig. 1. Curves showing change in body weight of rats receiving diets containing the following different combinations of protein and carbohydrate. A, fresh egg-white and wheat-starch; B, dried egg-white and potato-starch; C, dried egg-white and wheat-starch.

*The results of using egg-white dried in the laboratory.* The next step was to prepare a sample of dried egg-white in the laboratory. Fresh egg-white was placed in thin layers in flat porcelain dishes in a hot room maintained at 37° and a stream of air blown over it by means of an electric fan for from 12 to 24 hours. In this way a dry flaky undenatured substance was obtained. When this was dissolved in water and used as the protein in the DW diet, exactly the same symptoms resulted as when the Chinese preparation was

used. Thus it was demonstrated that when dried by evaporation at 37°, egg-white suffers some radical change in its nutritive properties which renders it unsuitable for use as the sole protein of the diet of young rats unless potato starch (or some other food substances) are ingested at the same time. Two alternative hypotheses seemed feasible to account for this fact.

(1) The dried egg-white contained some toxic substance which was neutralised by the potato starch.

(2) The dried egg-white had been deprived of some essential dietetic factor which was supplied by the potato-starch and not by the wheat-starch. This could hardly be an amino-acid since the potato-starch was not supplying more than 2 mg. of nitrogen per day (in later experiments protection was obtained when only 0.5 mg. of nitrogen was added daily to the diet by the potato starch).

Whichever hypothesis should prove to be correct the presence of a protective factor in potato-starch must be postulated. This will be referred to as protective factor X.

#### *Experimental details.*

The problem seeming one of fundamental interest, a lengthy investigation was undertaken. All the experiments were carried out on litters of rats at the age of 3 weeks, and in all cases control rats from the same litter were included. The date on which the first signs of soreness appeared at the corners of the mouth was noted, and this fact, the weight curve and the general condition of the rats were adopted as criteria of the experiments. In many experiments male rats only were used as they were found to give a quicker and more regular response than the females. Owing to the fact that potato-starch is extremely indigestible to rats, it was found necessary to steam all diets containing more than 20 % of the carbohydrate in this form.

The general results of the experiments are given below. Since experiments involving over 600 rats were carried out, space does not permit them to be described in detail, but curves from representative cases are given (Figs. 1, 2, 3 and 4).

#### *The result of variations in the relative amount of dried egg-white in the diet.*

In order to test the first of the two above hypotheses a series of experiments was planned in which diets containing varying concentrations of dried egg-white were used. If a toxic substance were the cause of the symptoms, a decrease in their severity should result from the ingestion of smaller quantities of dried egg-white, while on the other hand an increase in the relative consumption of the egg-white should be followed by the development of a more acute form of the disease. Diets containing approximately 10, 20 and 40 % of their dry weight as dried egg-white were used. The results obtained did not offer support to this theory, for whichever diet was used the condition

was equally severe and appeared as soon. This would however be in accord with the hypothesis that the rats were suffering from a more or less complete deficiency of some essential factor.

*The influence of variations in the method of desiccation.*

(1) *Reaction.* It was found that the reaction of the egg-white had no influence on the change which took place during desiccation, for if sufficient acetic acid were added to raw egg-white to render it acid to litmus, it still underwent the same change during subsequent dehydration.

(2) *Heat coagulation.* The experiments of Mitchell, referred to above, suggested that if the egg-white were first coagulated by heat, the drying process would be harmless. This proved to be correct, and suggested that the change taking place when egg-white is dried at 37° might be the work of a thermolabile enzyme acting at blood-heat. This idea was however disposed of by the discovery that the egg-white could be exposed in thin layers to the temperature of 37° without undergoing any change in its nutritive properties, provided that desiccation was prevented by the use of wet cloths stretched over the dishes.

(3) *Desiccation in vacuo.* When egg-white is dried *in vacuo* over  $H_2SO_4$  at room temperature the change still takes place, indicating that it is not an oxidation nor the result of exposure to 37°.

(4) *Dehydration by alcohol.* Some egg-white was dehydrated by treatment with successively greater concentrations of alcohol, and it was found that in this case also the egg-white had been damaged. Egg-white dried in this way was denatured during the process.

*The protective factor X in potato and other starches and in whole potato.*

The protective factor was found present in arrowroot as well as in potato-starch, while the cereal starches—rice and maize—were found to resemble wheat in being devoid of it. Potato-starch seemed to be rich in the factor, for a daily ration of 0.2 g. of raw starch was sufficient to protect rats fed on the DW diet from all the usual symptoms. Raw potato (Fig. 2) proved also a rich source of the factor, protection being given by 1.0 to 1.5 g. equal to 0.2 to 0.3 g. potato-starch per day. Fresh potato juice however was entirely inactive even when large amounts were ingested. The protective factor X therefore is probably so strongly adsorbed as to appear to be insoluble in water. This agrees with the fact mentioned above that raw potato does not contain more of the protective factor than the corresponding amount of potato-starch.

Attempts to deactivate potato-starch by extraction with alcohol and ether were unsuccessful.

*The effect of heat and desiccation upon the protective factor X.*

Some, though not all, of the protective power of potato is lost when potato is cooked by boiling or steaming. There is also evidence that destruction occurs when potato-starch is mixed with water and steamed. It was mentioned earlier in this communication that the diets containing large amounts of potato-starch were steamed before consumption in order to render them less indigestible. As these diets supplied amounts of the potato-starch far above the necessary minimum, the deleterious effect of heat upon the factor X was not apparent in these cases.

Desiccation at 37° does not appear to damage the protective factor in raw potato to any appreciable extent.

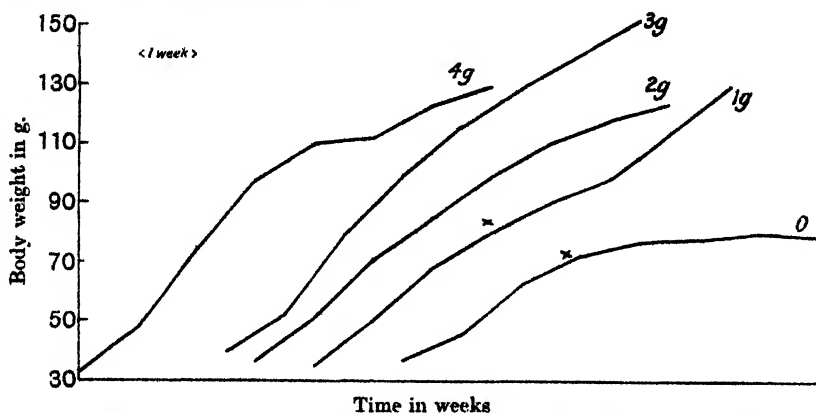


Fig. 2. Curves showing the change in body weight of male rats receiving the DW diet and a daily ration of raw potato. Each curve represents average figures for two or three rats. At x the symptoms of skin lesion appeared.

*The question of roughage.*

It was noticeable that when rats received potato-starch or raw potato in the diet they produced faeces many times more bulky than those of rats fed upon the egg-white diet prepared with wheat-starch. This bulkiness was due to the presence of undigested starch. If the dried egg-white were supposed to contain some toxic substance, the protective action of the starch might be due simply to mechanical removal of this substance from the intestine before complete absorption could take place. To test this theory agar-agar equal to 10 % of the dry weight of the diet was added to the DW diet. Although by this means bulky faeces were produced, the appearance and course of the usual symptoms were unaffected.

*The effect of desiccation upon other proteins.*

It was of interest to ascertain whether proteins other than those contained in egg-white suffered a like change when dried. It is quite evident that this is not the case of the vast majority of proteins, seeing that these are commonly dried and preserved in the dry condition before being incorporated in the diet, and can then be used as the sole source of protein

with complete success. In blood-serum the proteins are present in colloidal solution as they are in egg-white, and it therefore seemed possible that if horse-serum were dried in the same way as egg-white it might suffer a similar change in its nutritive properties. This did not prove to be the case. Moreover, the dried serum was found to contain the protective factor.

*The effect of supplementing with other proteins.*

At this stage in the investigation an incident occurred which, although unfortunate at the time, yet gave information of interest. I was unable to obtain any more Chinese egg-white from the firm which had up to then supplied it. A sample from a different source was tested and found to give the usual results. A larger supply was then obtained from this source and used for a series of experiments, but the results were irregular and unsatisfactory. Although some symptoms of the disease were obtained, in many cases they were only transitory, and followed by spontaneous cures. Only in few cases was the disease at all severe. This egg-white, unlike the first sample, was not entirely soluble in water, as (owing presumably to having been dried at too high a temperature) some of it had become denatured during the drying process. It had already been found that egg-white previously denatured by heat was not damaged by desiccation (p. 716) and the irregular results obtained with this partially denatured specimen suggested that denatured dried egg-white (and presumably fresh egg-white) was able to counteract the deleterious effects of the dried egg-white. If this were also true of fresh egg-white, the facts could be explained by the hypothesis that fresh egg-white contains a dietetic factor not present in the rest of the diet, this factor being destroyed during desiccation, but preserved by heat denaturation.

To test this theory the following experiment was carried out. Six male rats of the same litter were divided into three groups at the age of 21 days. Group A received the ordinary DW diet in which the dried egg-white formed 20 % of the dry weight, and the other groups received the same diet modified in two different ways. In group B the dried egg-white was reduced to 10 % of the dry weight without any addition. In group C the protein of the diet consisted of 10 % dried egg-white and 10 % fresh egg-white.

The curves of increase in body weight of these rats are shown in Fig. 3. In the first two groups the usual symptoms developed but in group C the fresh egg-white was able to give protection. It appeared that this was the minimum amount which would give this result, for after receiving the diet for 3 weeks all the rats showed a slight soreness at the corners of the mouth which lasted for a few days and then disappeared.

*The storage of the protective factor X by rats.*

In the spring and summer of 1926, the rats receiving the DW diet began to show a greater variability than those of previous work. Some rats or even some whole litters exhibited an unusual resistance to the DW diet. Finally

in September three separate litters which were receiving the DW diet grew well and showed only slight signs of skin affection even after they had received the DW diet for 6 to 7 weeks. There was no variation in the sample of dried egg-white or in the wheat-starch used, and it seemed possible that the rats might in some way have themselves acquired greater reserves of the protective factor than was usual. This idea was based on the assumption that a storage of the factor could take place as has been demonstrated in the case of some of the other accessory factors.

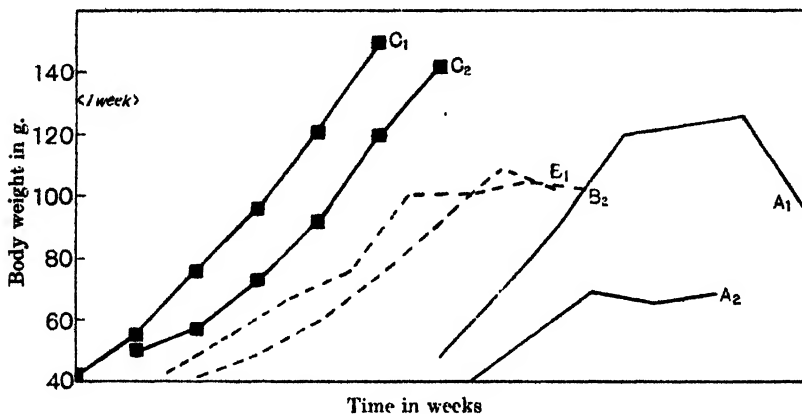


Fig. 3. Curves showing the change in body weight of 6 male rats of the same litter.

Rats A<sub>1</sub> and A<sub>2</sub> received the DW diet.

Rats B<sub>1</sub> and B<sub>2</sub> received the DW diet modified by the reduction of the protein to one-half the usual amount.

Rats C<sub>1</sub> and C<sub>2</sub> received the DW diet modified by the replacement of one-half of the protein by fresh egg-white.

All the rats used had been bred in the Institute on a diet of milk, white and brown bread, marmite, cabbage, carrot, raw meat and various grains. During the past year the amount of water-soluble vitamins in the diet had been increased, in particular by the addition of large amounts of rolled oats and of wheat embryos. It was arranged that some pregnant does should receive a restricted diet during the last week or so of pregnancy and throughout lactation. This diet consisted of white bread, milk, cabbage, carrot and meat. Some litters which did not appear to thrive received a little brown bread occasionally. Although on this diet well-grown healthy litters were obtained it proved a severe strain on the mothers. They were often in such poor condition at the end of the lactation period that they could no longer be used for breeding purposes. I also obtained some litters of rats from an outside source bred on a diet inferior to that of the Institute stock.

Young rats from these two sources were fed upon the DW diet and the results were compared with those obtained with litters the mothers of which had received the full stock diet during lactation. An analysis was made of the condition of the three groups of rats after they had received the experimental diet for 5 weeks. A note was made as to whether the body weight

was rising, falling or stationary and a number of crosses indicated the physical condition of the animal.  $\times$  indicated no definite symptoms except a rough coat and soreness round the mouth.  $\times\times$  meant definite dermatitis and loss of hair but unaccompanied by nervous trouble, and when  $\times\times\times$  was given both dermatitis and nervous symptoms were severe.

When analysed in this way the results of the experiments showed that the *rapidity* with which the disease developed in young rats was controlled by two things:

- (a) the weight of the rat at the beginning of the experiment;
- (b) the diet received by the mother during pregnancy and lactation.

When rats of equal initial body weight were compared, those whose mothers had been fed upon the restricted diet showed a more advanced stage of the disease after they had received the DW diet for 5 weeks than did those from mothers fed upon the usual stock diet.

It appears therefore that rats are able to store the protective factor and that the degree of this reserve in young rats depends not only on their own body weight but also on the amount of the factor in their own previous diet and in the diet of the mother. When possessing large reserves of the factor the young rat can grow well and have an almost normal skin and coat for many weeks on the DW diet, but eventually the reserves are used up and the disease appears and takes its usual course. In an experiment with adult rats of 200–300 g. on the DW diet three months elapsed before the symptoms appeared.

*The connection between the protective factor X and the  
water-soluble vitamins.*

The above experiments upon the storage of the factor showed that when the water-soluble vitamin content of the diet was reduced there was an accompanying decrease in the amount of the unknown protective factor. It seemed possible that there might be some connection between this factor and the active principles contained in McCollum's vitamin B, more especially as they possess a similar distribution in many substances, notably egg-white and raw potato. They could not be identical, for potato-starch, one of the most potent sources of the protective factor, is devoid of vitamin B, although from Eijkman's work [1906] it appears to possess some antineuritic power. Further, marmite, which is capable of supplying the water-soluble vitamins, did not contain the protective factor. The work of Goldberger and his co-workers [Goldberger, Waring and Tanner, 1923; Goldberger and Tanner, 1924, 1925; Goldberger, Wheeler and Tanner, 1925; Goldberger, Wheeler, Lillie and Rogers, 1926; Goldberger and Lillie, 1926] was of great interest in this connection. Most of the work was done on cases of human pellagra. Goldberger demonstrated that water-soluble B consisted of two different factors, one the antineuritic or antiberiberi, and the other the pellagra-preventive. He found that the first was more easily destroyed by heat than was the second, for

yeast autoclaved at  $120^{\circ}$  for 5 to 6 hours was found to have retained its curative power towards pellagra, although the antineuritic potency was lost. Although often found together in nature, the two factors did not show an identical distribution, wheat germ for example being much richer in the antineuritic factor than in the pellagra-preventive.

The rats fed upon the DW diet showed symptoms which in many ways resembled those of pellagra, and it seemed of interest to determine the content of the protective factor X in those substances which Goldberger had found to be rich in his pellagra-preventive factor. Table I shows roughly the content of the protective factor X and of the supposed two constituents of the water-soluble vitamins in a number of foodstuffs. It will be seen that all three are present in dried yeast (Fig. 4); when the yeast is heated for a long time at a high temperature (4 to 5 hours at  $120^{\circ}$ ) the antineuritic vitamin is destroyed but the other two are unaffected. Milk also contains all three, which are therefore found as impurities in commercial caseinogen. Extraction of the caseinogen with alcohol removes the B vitamins but not the protective factor X.

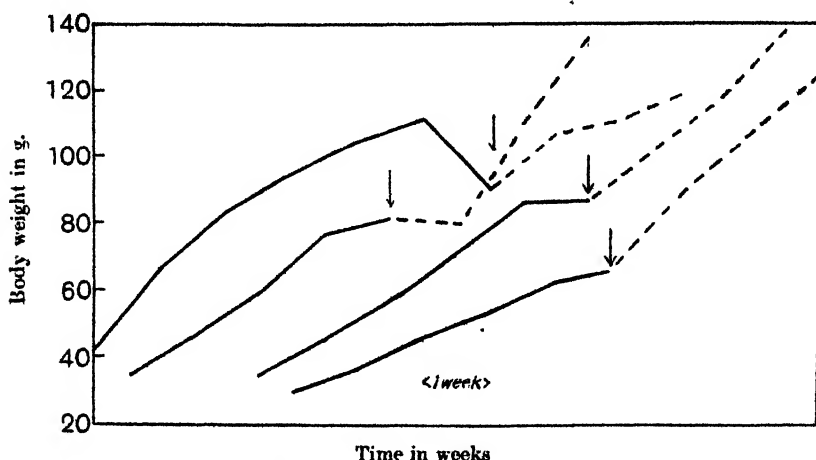


Fig. 4. Curves showing the change in body weight of rats fed upon the DW diet. At the arrows daily rations of dried yeast (0.2 to 0.4 g.) were given in addition.

Raw potato contains both the antineuritic vitamin and the protective factor (it has not yet been tested for the pellagra-preventive). The protective factor remains associated with the starch throughout its preparation, while the antineuritic is present in potato juice and is washed out of the starch.

Thus it appears that the protective factor X, though having in many ways a similar distribution to vitamin B, does not coincide with either of its supposed constituents. This is confirmed by the nature of the symptoms produced when the protective factor is absent from the DW diet. The nervous symptoms (spasticity of the limbs) are not the same as the paralysis induced in rats by deprivation of the antineuritic factor, nor are the skin lesions identical with



those observed in cases of experimental pellagra in rats as described by Goldberger [Goldberger and Lillie, 1926] and confirmed by Chick and Roscoe [1927]. Rats deprived of the pellagra-preventive factor by these workers developed scaly thickened ears and inflamed oedematous feet, whereas the rats in my experiments never showed the first and only in a few cases the second.

Table I.

Showing the distribution in certain substances of the two constituents of vitamin B and of the protective factor X. The figures for the antineuritic vitamin are taken from the Medical Research Council's Report on the Present State of Knowledge of Accessory Food Factors [1924] and those for the pellagra-preventive factor from Goldberger's work and from the work of Chittenden and Underhill [1917] and Underhill and Mendel [1925] on "black-tongue," a pellagrous-like condition in dogs.

Substance	Protective factor X	Water-soluble B of McCollum	
		Eijkman's anti-neuritic	Goldberger's pellagra-preventive
Raw potato ... ..	+++	++	
Cooked potato ... ..	+	+	
Dried potato ... ..	++	++	
Potato-starch ... ..	+++	+ (Eijkman)	
Cooked potato-starch ...	+		
Potato-juice ... ..	0	++	
Arrowroot ... ..	+++	0	
Cereal starches ... ..	0	0	0
Dried yeast ... ..	+++	+++	+++
Autoclaved yeast ... ..	++	0	++
Marmite ... ..	0	++	++
Harris vitamin B concentrate	?	+	++
Fresh egg-white ... ..	+	0	
Dried egg-white ... ..	0	0	
Dried coagulated egg-white	+	0	
Dried egg-yolk ... ..	++	+	
Fresh milk ... ..	+	+	+
Commercial caseinogen ...	+	++	+
Crude lactalbumin ... ..	+	+	+
Extracted heated caseinogen	+	0	0
Dried horse-serum ... ..	+		
Wheat-germ ... ..	?	++	+
Wheat-bran ... ..	0	+	
Wheat-flour ... ..	0	0	0
Wheat-starch ... ..	0	0	0
Wheat-gluten ... ..	+		
Malt-extract ... ..	0	+	
Raw meat ... ..	0	+	++
Fresh spinach ... ..	+		
Fresh cabbage ... ..	+	+	
Banana ... ..	+	+	
Butter ... ..	0	0	+ Black tongue

### DISCUSSION.

In the earlier stages of the investigation it seemed possible that the phenomena observed might be explained by one of the following two alternative hypotheses.

(1) When fresh crude egg-white is dried an essential dietetic factor in it, the existence of which has been hitherto unrecognised, is destroyed. In the absence of this factor from a diet in which the nitrogen is entirely supplied by the egg-white and is complete in all other respects young rats develop a

characteristic universal dermatitis accompanied by nervous disorder which is finally terminated by death. This factor X is present in fresh eggs, both in the yolk and in the white, in dried yeast, raw potato, potato-starch and arrowroot, milk, blood-serum, banana and in fresh spinach and cabbage leaves.

(2) Some toxic product formed when fresh egg-white is dried is responsible for the symptoms which develop when rats are fed on a diet containing dried egg-white as the sole source of protein. The toxic substance can be neutralised by a factor X present in the foodstuffs enumerated in hypothesis (1).

The investigation has not yet reached a stage at which it is possible to decide which, if either, of the above hypotheses is correct. Neither seems entirely satisfactory. So far as at present investigated, egg-white is the only crude protein mixture which is susceptible to this deterioration of its nutritive properties on drying. Dried horse-serum, commercial preparations of caseinogen, gluten and lactalbumin, when they were the only proteins supplied, did not produce the symptoms which were shown by rats receiving a diet in which dried egg-white was the sole protein.

Many facts of the problem which appear irreconcilable may ultimately prove to be due to differences in the amount of the factor X present. Suppose egg-white to contain only just sufficient of it, then a partial destruction of the factor during desiccation would result in the appearance of the symptoms, whereas caseinogen, serum, etc., might contain so much that the loss of a part of it would still leave sufficient for the needs of the animal.

Experiments now in progress however indicate that there is a relationship between the amount of dried egg-white ingested and the amount of the protective factor X needed to give protection.

It seems therefore probable that the true explanation of the problem is a more complicated one than either of the two above theories. It may have some relation to the problem studied by Hartwell [1924, 1, 2; 1925] and also recently by Reader and Drummond [1926]. They find that a dietary factor having a similar distribution to vitamin B was able to correct a diet overbalanced by excess of protein. Since marmite contains this factor, it cannot be the same as the protective factor X, but it is quite conceivable that in the problem set out in this paper a similar question of balance between two constituents of the diet is involved.

#### SUMMARY.

(1) A diet of crude egg-white, boiled and supplemented with wheat-starch, cotton-seed oil, cod-liver oil, lemon juice, marmite, salts and water, supports young rats in growth and health.

(2) If the egg-white is previously dried the diet is inadequate unless the carbohydrate is supplied in the form of potato-starch or arrowroot, or unless certain substances are added in small amounts.

(3) The condition which is developed by rats fed on this unsatisfactory diet is described in detail.

(4) The change which takes place in dried egg-white is independent of the reaction of the solution during drying.

(5) It is not the work of a thermolabile enzyme.

(6) It does not appear to be a process of oxidation.

(7) Egg-white previously coagulated by boiling is not damaged by desiccation.

(8) The crude proteins of horse-serum and milk do not suffer a similar change during desiccation.

(9) The foodstuffs which possess the power of counteracting the ill-effects resulting from the ingestion of the dried egg-white are raw potato, potato-starch, arrowroot, dried yeast, fresh egg-white, egg-yolk, milk, commercial caseinogen, crude lactalbumin, spinach and cabbage leaves, banana, and dried horse-serum. The presence of a protective factor X in these substances is postulated.

(10) This factor shows a similar distribution in many ways to that of the water-soluble B vitamins. It is not however identical with either the anti-neuritic factor or Goldberger's pellagra-preventive.

(11) Its resistance towards heat and desiccation varies according to the substance in which it is found.

(12) The resistance shown by rats to the ill-effects of the dried egg-white diet depends chiefly on the body weight of the animal at the beginning of the experiment. It is also influenced by the diet of the mother during pregnancy and lactation. It seems probable therefore that rats can store reserves of the protective factor X.

I should like to record my gratitude to Sir C. J. Martin and Dr H. Chick for their unfailing help and advice throughout the course of this investigation.

#### REFERENCES.

- Boas (1924, 1). *Biochem. J.* **18**, 422.  
 — (1924, 2). *Biochem. J.* **18**, 1322.  
 Bond (1922). *Biochem. J.* **16**, 479.  
 Chick and Roscoe (1927). *Biochem. J.* **21**, 698.  
 Chittenden and Underhill (1917). *Amer. J. Physiol.* **44**, 13.  
 Eijkman (1906). *Arch. Hyg.* **58**, 49.  
 Goldberger and Lillie (1926). *U.S.A. Public Health Report*, **41**, 1924.  
 Goldberger and Tanner (1924). *U.S.A. Public Health Report*, **39**, 87.  
 — (1925). *U.S.A. Public Health Report*, **40**, 54.  
 Goldberger, Waring and Tanner (1923). *U.S.A. Public Health Report*, **38**, 2361.  
 Goldberger, Wheeler, Lillie and Rogers (1926). *U.S.A. Public Health Report*, **41**, 297.  
 Goldberger, Wheeler and Tanner (1925). *U.S.A. Public Health Report*, **40**, 927.  
 Hartwell (1924, 1). *Lancet*, ii, 956.  
 — (1924, 2). *Biochem. J.* **18**, 785.  
 — (1925). *Biochem. J.* **19**, 1075.  
 McCollum, Simmonds and Pitz (1917). *J. Biol. Chem.* **29**, 521.  
 Medical Research Council (1924). *Special report series*, No. 38.  
 Mitchell (1925). *Amer. J. Physiol.* **64**, 359.  
 Osborne and Mendel (1913, 1). *J. Biol. Chem.* **15**, 311.  
 — (1913, 2). *J. Biol. Chem.* **16**, 423.  
 — (1915). *J. Biol. Chem.* **20**, 351.  
 Reader and Drummond (1926). *Biochem. J.* **20**, 1256.  
 Underhill and Mendel (1925). *U.S.A. Public Health Report*, **40**, 1087.





# **XCVI. A NOTE ON THE VITAMIN D CONTENT OF THE STOMACH OIL OF THE AUSTRALASIAN PETREL (*AESTRALATA LESSONI*).**

By JOAN LEIGH LEIGH-CLARE.

*From the Department of Experimental Pathology, Lister Institute, London.*

*(Received May 2nd, 1927.)*

THE Australasian petrel (*Australata lessoni*), or mutton bird, flies North from the antarctic regions during the summer months, to the islands of the South Pacific, where mating and breeding take place. Here its staple diet consists of "whale-feed," vast shoals of which are met with throughout the temperate seas of the southern hemisphere.

This "whale-feed," according to G. M. and G. S. Thomson [1923] consists largely of a bright red shrimp, which is a swimming stage in the life-history of the crustacean *Munida gregaria*. Carter and Malcolm [1926] state that this crustacean is also the principal food of the Australasian red cod at this time of the year.

Mutton bird oil is obtained from the stomach of the young petrel, and is commonly believed to be supplied to the young from the stomachs of the parent birds. It is stated by Carter [1921] to consist for the most part of cetyl oleate and other esters of cetyl alcohol. As, however, Carter and Malcolm [1926] found no trace of cetyl alcohol in the oil extracted from "whale-feed," it is obvious that mutton bird oil is not the fatty residue from the digestion of the latter. Its origin is mysterious. Rosenheim and Webster [1927] suggest that it is a special secretion used for oiling the feathers by the agency of the bird's beak. Comparative growth tests have been made on this oil and cod-liver oil by Malcolm [1926] who pronounced it to be "one of the richest known natural sources of vitamin A."

Rosenheim and Webster [1927], however, point out that this statement requires modification, since in his animal tests Malcolm did not give a constant supply of vitamin D. The above-named workers were unable to obtain a supply of mutton bird oil. They therefore examined the stomach oil of the fulmar petrel (*Fulmarus glacialis*) which breeds in enormous numbers in St Kilda, and which corresponds most closely to the antarctic mutton bird. They found that when a constant supply of vitamin D was given throughout the experiment, the vitamin A content was no better than that of a good Newfoundland cod-liver oil.

These workers also demonstrated the presence of vitamin D in the stomach oil of the fulmar petrel, and found that "a dose of 20 mg. gave partial protection, whilst 40 mg. completely protected the animals against rickets."

By the kindness of the African Industries Trade Service, some mutton bird oil was obtained for the following experiments.

Two litters of rats were used. No. 1 was a litter of six white rats weighing 39-50 g., and No. 2 a litter of twelve black-and-white rats, weighing 36-46 g. at the beginning of the experiment. The basal diet given was McCollum's No. 3143 low phosphorus, rickets-producing diet. The doses of mutton bird oil were given daily from the start of the experiment. A series of control animals receiving various doses of a good cod-liver oil was included in order to provide a positive control for the experiment. As will be seen from the table, the doses were given in multiples of five, starting from a daily dose of 0.2 mg. of the petrel oil and culminating in a dose of 120 mg.

Litter No. 2 was killed at the end of 23 days and litter No. 1 at the end of 24 days. To test the condition of the skeletons of the rats, the junctions of the 6th and 7th ribs on the right side were examined histologically, and chemical analyses were made of the leg-bones (femora, tibiae and fibulae). The methods used are described in full in the paper by Chick, Korenchevsky and Roscoe [1926].

In comparing the effect of the two oils, both the chemical and histological analyses indicate that petrel stomach oil has a vitamin D value equal to about 1/5 of that of the cod-liver oil. Doses of the former of 5, 25 and 120 mg. afforded respectively about the same degree of protection as 1, 5 and 25 mg. of the latter. For the rats receiving the above three doses of petrel oil the average value of the A/R ratio (ratio of the mineral ash to the organic matter in the fat-extracted bones) was 0.64, 0.74 and 0.92 respectively; for rats receiving the above-stated cod-liver oil doses, it was 0.59, 0.81 and 0.93. Similarly, the mean values of the percentage of ash contained in the fat-extracted dry bone were 39.1 and 43.8 for animals receiving respectively daily doses of 5 and 25 mg. petrel oil and 39.6 and 44.6 for those receiving cod-liver oil in doses of 1 and 5 mg. respectively (see table).

Rat	Litter	Sex	Oil	Dose (mg.)	A/R	Average	% ash in fat-extracted dry bones	Degree of rickets, from histology of rib junctions
31	1	♂	Petrel	0.2	0.474	0.50	31.75	Severe rickets
37	2	♀	"	0.2	0.632		38.74	
33	1	♂	"	1.0	0.461	0.45	31.55	Severe rickets
39	2	♀	"	1.0	0.443		35.77	
40	2	♂	"	1.0	0.448		38.94	
34	1	♂	"	5.0	0.639	0.64	38.99	Severe rickets
43	2	♀	"	5.0	0.647		39.28	
35	1	♂	"	25.0	0.548	0.74	40.84	Severe rickets
44	2	♀	"	25.0	0.801		44.48	
47	2	♂	"	25.0	0.858	0.92	46.17	Osteoporosis only
48	2	♀	"	120.0	0.920		47.91	
32	1	♀	Cod	1.0	0.535	0.59	34.84	Severe rickets
38	2	♀	"	1.0	0.750		42.86	
41	2	♂	"	1.0	0.486		41.18	
42	2	♀	"	5.0	0.806	0.81	44.62	Slight rickets and osteoporosis
36	1	♂	"	25.0	0.962	0.93	49.03	Osteoporosis only
45	2	♀	"	25.0	0.932		48.23	
46	2	♂	"	25.0	0.904		47.47	

The histology of the rib junctions of animals receiving 5 mg. of petrel oil or 1 mg. of cod-liver oil showed decided rickets. This condition was much modified when the daily doses of the oils were increased to 25 mg. of petrel and 5 mg. of cod-liver oil respectively, and where the daily ration was further increased to 120 mg. of petrel or 25 mg. of cod-liver oil, osteoporosis only was seen.

#### SUMMARY AND CONCLUSIONS.

The presence of vitamin D in amount equal to about 1/5 of that present in a good cod-liver oil, has been demonstrated in the stomach oil of the Australasian mutton bird (*Australata lessoni*).

How the vitamin D came into the stomach oil is unknown. This oil appears to consist mostly of cetyl esters and therefore not to be the undigested fatty residue from the "whale-feed." However, the fact that the mutton bird feeds on the same crustacean ("whale-feed") as the Australian red cod, seems to suggest that this might be the common source from which both bird and fish draw their supplies of vitamin D.

My thanks are due to Prof. C. J. Martin, Dr Chick and Prof. Korenchevsky, for their kind help and criticism, to the African Industries Trade Service for the sample of petrel oil used in the experiments, to the Medical Research Council for a whole time grant and to the Lister Institute for the hospitality of its laboratories.

#### REFERENCES.

- Carter (1921). *J. Soc. Chem. Ind.* **40**, 2201.  
Carter and Malcolm (1926). *Trans. N. Zealand Inst.* **56**, 647.  
Chick, Korenchevsky and Roscoe (1926). *Biochem. J.* **20**, 622.  
Malcolm (1926). *Trans. N. Zealand Inst.* **56**, 650.  
Rosenheim and Webster (1927). *Biochem. J.* **21**, 111.  
Thomson, G. M. and G. S. (1923). *N. Zealand J. Sci. Tech.* **16**, 111.



# XCVII. THE SYNTHESIS OF THE ANTINEURITIC FACTOR (TORULIN) BY YEAST.

By FRANK HAWKING.

*From the Rockefeller Department of Biochemistry, Oxford.*

*(Received May 4th, 1927.)*

THERE has been some uncertainty as to whether yeast could synthesise vitamin B when grown on a medium which did not contain it. Macdonald [1922], Heller [1923], and Nelson, Fullmer and Cessna [1921] tested for the factor promoting growth in rats and found it present. Harden and Zilva [1921] and Heller [1923] tested for the antineuritic factor, and found this to be synthesised. But Eijkman, Hoogenhuijze and Derks [1922] found that the antineuritic factor did not occur in yeast unless the medium contained either it or its decomposition products.

In all these experiments there seems to have been no test at the end as to the success of the precautions against bacterial contamination, although it is known that some species of bacteria can synthesise vitamin B [Damon, 1923; Heller, Elroy and Garbeck, 1925]. In some of the experiments it is almost certain that unsuspected impurities containing bios were present [Hoet, le Clef and Delrue, 1924; Funk and Freedman, 1923; Willaman and Olsen, 1923]. Hence further experiments were undertaken to investigate whether yeast could synthesise the antineuritic factor, if the possibility of active impurities in the medium and of bacterial contamination was excluded or controlled. The antineuritic factor is considered here to be the factor curative for symptoms of head retraction in pigeons fed upon polished rice.

## *Method.*

The medium used was:

$\text{KH}_2\text{PO}_4$	...	...	5 g.	Cane sugar	...	50 g.
$\text{NH}_4\text{Cl}$	...	...	2.5 g.	Water	...	1 litre
$\text{MgSO}_4$	...	...	0.35 g.	Bios extract	...	3 cc.
$\text{CaCl}_2$	...	...	0.25 g.			

The cane sugar was recrystallised three times from 80 % alcohol. The salts were either purified by double recrystallisation, or Kahlbaum's preparations were used. All glassware was cleaned with chromic acid. The water used was glass-distilled from alkaline permanganate.

The bios extract deserves further explanation. It was prepared by Mr G. L. Peskett from baker's yeast, according to the method of Eddy, Kerr, and Williams [1924], up to the "fuller's earth" stage, and its activity may be

# SYNTHESIS OF THE ANTINEURITIC FACTOR BY YEAST 729

judged from the following experiment (Table I). Flasks containing 100 cc. of the salt solution and various concentrations of the extract were inoculated, and after growing for 6 days at 33° the yeast was centrifuged off, dried at 140° overnight, and weighed.

Table I.

Conc. of bios extract %	Dry wt. of culture g.	Wt. of yeast Conc. of bios
1.0	0.10, 0.15	0.125
0.5	0.10, 0.14	0.240
0.2	0.07, 0.10	0.085
0.1	0.04, 0.04	0.04
0.025	hardly any growth	—

The extract also contained a certain amount of vitamin. The attempt was made to control this by using low concentrations of extract in the medium. The content of vitamin may be obtained from the following results (Table II), obtained by the curative and protection test [Kinnersley and Peters, 1925].

Table II.

Pigeon	Dose of extract cc.	Duration of cure
1	1	No cure
2 (re-treated bird)	5	2 days
3	5	7 "
4	4.5	6 "
5	4.5	1 day

Prof. Peters makes the following comment upon the figures.

"The bios extracts are distasteful to the birds and the results obtained exceptionally variable. This is apt to be so with inactive concentrates, and it is usually found that re-treated birds react less readily to curative extracts than birds fresh from the dealers. For the purpose of this paper it would appear wisest to take the least favourable results, namely, experiments 3 and 4."

Hence, for the purpose of this paper, it may be concluded that the extract contained not more than 1.4 day doses per cc., i.e. 2.1 day doses per 500 cc. medium as a maximum.

The strain of yeast used was *Saccharomyces cerevisiae* Nat. Type Culture No. 381/21, from the Lister Institute. One drop of a 48 hours' growth in the above medium was transferred to 10 cc. of the salt solution, and one drop of this was used to inoculate each of the 500 cc. culture flasks. The inoculation contained about 20,000 cells. The flasks were incubated for 5 days at 30–33° and then the yeast was centrifuged off and extracted twice with 70 % alcohol as described by Funk, Harrow and Paton [1923].

Before centrifuging, smears were made of the yeast grown and subcultures of the supernatant fluid were also made on to agar slopes. These were incubated for 2 days at 33° and one at 37°, and then a composite smear was made from

each tube. The two sets of slides thus obtained were examined for bacterial contamination, staining with carbol-thionine blue. Careful inspection showed it slight, but an attempt was made to give some objective indication of the degree to which it was present. Three microscope fields on each slide were taken at random, and everything that might possibly be a micro-organism was counted, the result being expressed as a percentage of the number of yeast cells present in the same field (300-500). When expressed thus, the contamination of the actual yeast extracted was about 1-2 % (see below). This is an outside figure and is probably excessive, especially as the agar slopes—presenting conditions favourable to bacterial growth—gave a figure only slightly higher (2-3 %). In any case, the proportion by volume was very much less, since a yeast cell is at least 250 times as large as an average micro-organism.

### RESULTS.

A series of earlier experiments was spoilt by the presence of too much bios extract, and so, of too much vitamin in the culture medium. The latest results obtained with the most improved technique were as follows:

Table III.

Pigeon	Yeast wt. g.	Cured or protected for days	Contamination	
			Smear %	Agar slope %
5	3.7	3	1.3	—
6	3.9	5	1.3	3.0
7	3.85	3+	1.5	2.2
8	2.95	7	2.1	—

Pigeon 8 came from an irregular batch of which several other birds gave abnormally high results, and must therefore be discarded. For the purpose of the experiment, pigeons 5 and 7 gave the lowest result, namely, 3 days. If this lowest figure is therefore taken for the curative effect of the yeast, and compared with the highest figure obtainable for the amount of possible vitamin in the bios present in the medium, the results obtained will be the most unfavourable to synthesis found under the conditions of the experiments. The facts are that not more than 2.1 day doses/500 cc. were originally present, whereas not less than 3 day doses were found in the yeast after growth. A balance of synthesis of 1 day dose out of three is therefore indicated.

### DISCUSSION.

The increase of activity observed is small, and it might be suggested that it could be attributed to unrecognised impurities present in the medium. Though the salts and sugar had been purified as described, an attempt was made to extract the medium with successive fractionations with 70 % alcohol. This had to be abandoned owing to the difficulty of freeing from sugar sufficiently to give to a bird. Against the possibility of slight contamination in the medium, however, may be set the probability that Funk's method does

## SYNTHESIS OF THE ANTINEURITIC FACTOR BY YEAST 731

not extract all the torulin present. Further, no allowance can be made for a possible utilisation of the factor by the yeast in the course of its metabolism. The figures given for the bacterial contamination which are maximal suggest that this possible source may be neglected.

A general consideration of the problem of synthesis by the yeast cell suggests that such synthesis is likely to be small unless a much purer bios preparation can be obtained. It is inherently probable that an optimal concentration of the vitamin in the yeast cell can be obtained either by intracellular synthesis or adsorption from the environment. Fluid in which yeast has grown is notably free from vitamin, a fact which was confirmed in some of the earlier experiments. Two lots of 300 cc. of medium, after growth of the yeast, were extracted with 66 % alcohol and taken up in aqueous solution but failed to cure birds, although initially 4 day doses were present in each. Adsorption of vitamin by the yeast has been stressed by Eijkman *et al.* [1922], Randoïn and Lecoq [1926] and Southgate [1924]. It may be concluded that yeast preferentially adsorbs the factor, merely synthesising when necessary to meet the optimal requirements. Therefore until a bios preparation can be obtained containing a growth-promoting factor of the bios type in much larger concentration than it does torulin or its decomposition products, the problem would seem impossible of final settlement. There were two other contentions, contained in Eijkman's paper. It was suggested that yeast could not synthesise the antineuritic factor, but merely reactivated its decomposition products. In these experiments, treatment with alkali was avoided, and the method of preparation of the bios should not be destructive of torulin. It is, however, still possible that the apparent synthesis is merely a reactivation of some decomposition product. The further contention that, as the antineuritic factor is not synthesised by yeast, it is to be distinguished from the growth-promoting factor, is puzzling to interpret in the light of recent work upon the duality of vitamin B. If both factors are needed for the growth of the rat, some synthesis of the antineuritic vitamin must have occurred.

### SUMMARY.

Evidence is produced to show that yeast (*S. cerevisiae*) can synthesise the curative factor for pigeons (torulin) in small amounts.

I wish to acknowledge my indebtedness to Mr G. L. Peskett for his invaluable advice in connection with the growth of yeast cultures, and to Mr W. H. Kinnersley for testing out the extracts on the pigeons.

## REFERENCES.

- Damon (1923). *J. Biol. Chem.* **56**, 895.  
Eddy, Kerr and Williams (1924). *J. Amer. Chem. Soc.* **46**, 2846.  
Eijkman, Hoogenhuijze and Derks (1922). *J. Biol. Chem.* **50**, 311.  
Funk and Freedman (1923). *J. Biol. Chem.* **56**, 851.  
Funk, Harrow and Paton (1923). *J. Biol. Chem.* **57**, 154.  
Harden and Zilva (1921). *Biochem. J.* **15**, 438.  
Heller (1923). *J. Biol. Chem.* **55**, 385.  
Heller, Elroy and Garbeck (1925). *J. Biol. Chem.* **65**, 255.  
Hoet, le Clef and Delrue (1924). *Arch. Int. Physiol.* **23**, 284.  
Kinnersley and Peters (1925). *Biochem. J.* **19**, 820.  
Macdonald (1922). *J. Biol. Chem.* **54**, 243.  
Nelson, Fullmer and Cessna (1921). *J. Biol. Chem.* **46**, 77.  
Randoin and Lecoq (1926). *Compt. Rend. Acad. Sci.* **182**, 1564.  
Southgate (1924). *Biochem. J.* **18**, 1248.  
Willaman and Olsen (1923). *J. Biol. Chem.* **55**, 817.

## XCVIII. ON OVOMUCOID.

By JOSEPH NEEDHAM.

*From the Biochemical Laboratory, Cambridge.*

*(Received April 29th, 1927.)*

### I. THE CARBOHYDRATE CONTENT OF THE OVOMUCOID MOLECULE DURING THE DEVELOPMENT OF THE CHICK.

WHEN the carbohydrate metabolism of the extra-embryonic part of the developing hen's egg was being considered in a previous paper [Needham, 1926], an attempt was made to gain some idea of the movements of the "ovomucoid" fraction by adding the glycogen-glucose to the free glucose and subtracting the value so obtained from the total glucose. The resulting curve (being admittedly only of exploratory significance) gave a peak in the middle of incubation between its initially high values and the minimal ones of the end of incubation. This conflicted with the results of Bywaters [1913], who by means of analyses of coagulable and non-coagulable nitrogen in the egg-white during incubation concluded that there was no preferential absorption of albumin or ovomucoid but that both diminished steadily in the egg-white as development proceeded. Assuming that Bywaters' technique was satisfactory, as may most probably be done, there could be only two explanations of this discrepancy, (a) that the ovomucoid molecule contained more glucose in its prosthetic group at some times than at others during incubation, or (b) that the hump in the ovomucoid curve was due to a temporary increase of mucoprotein in the yolk only, at the time in question, mucoprotein which might or might not possess the same amount of glucose within its molecule as the ovomucoid of the egg-white. The former possibility was made somewhat likely by the recent work of Komori [1926], who considers that the glucose of ovomucoid is united to the protein part in the form of a polysaccharide, but if it were true, the results of Bywaters who found the ratio

$$\frac{\text{g. uncoag. N per 100 g. egg-white}}{\text{g. total glucose per 100 g. egg-white}}$$

to be more or less constant during incubation, would have to be abandoned.

In order to settle this question, therefore, preparations were made of ovomucoid from the egg-white from the 5th to the 18th day of incubation. The method used was as follows. The eggs were candled and pencil marks made on the shells to indicate the clear part where the egg-white lay close to the shell-membrane. They were then opened in that region, and the egg-white was carefully collected, though no attempt was made to recover every trace. The chalazae were included with the white. The mass was then weighed in a

tared bottle, diluted to twice its volume with distilled water and placed on a shaking machine for 2 hours in order to break up the albumin. For this purpose a quantity of broken porcelain was added and a few drops of caprylic alcohol to prevent frothing. The mixture was then further diluted and heated in a boiling water-bath for an hour with acetic acid, the reaction being so adjusted as to remain green to bromocresol purple. In this way the albumin was almost completely removed, subsequent tests with trichloroacetic acid demonstrating the presence of only the slightest traces of albumin. The filtrate was next poured into ten times its volume of 97 % alcohol, and the flocculent white precipitate filtered off, dissolved in as small an amount of hot water as possible, and reprecipitated. It was finally dried well over calcium chloride in a vacuum desiccator, and eventually hydrolysed for 4 hours with 5 % hydrochloric acid, the glucose or glucosamine set free being estimated in the filtrate by the Hagedorn-Jensen method [1923] after precipitation with phosphotungstic acid, which does not remove glucosamine.

Table I. *Experiments on ovomucoid.*

1	2	3	4	5	6	7	8	9	10	11	12
Day	No. of egg- whites	Total wt. g.	Egg-white g. per egg	Col. 4 smoothed	Wt. of egg-white in % of whole egg	Pure ovomucoid isolated mg.	Col. 7 mg. per egg	Col. 8 smoothed	Ovomucoid in mg. % of wet egg-white	Mg. taken for esti- mation	% glucose
0	—	—	—	—	—	—	—	—	—	—	—
1	—	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—	—	—
4	—	—	—	—	—	—	—	—	—	—	—
5	32	387	12.1	11.8	21.15	3583	112	128	1080	453	11.40
6	31	340	10.9	11.6	21.02	774*	25	125	1080	254	8.44
7	36	418	11.7	11.5	20.95	380*	11	122	1060	380	7.38
8	25	278	11.1	11.3	20.77	3563	143	119	1053	643	13.35
9	19	218	11.5	11.0	20.40	1015	53	117	1065	345	7.99
10	17	180	10.6	10.6	19.74	1427	84	110	1039	447	6.40
11	14	150	10.1	10.0	18.80	960	69	103	1030	310	12.80
12	18	173	9.6	9.3	17.60	2405	134	92	989	785	9.39
13	9	73	8.2	8.3	15.85	584	65	82	988	414	12.00
14	29	335	7.6	7.1	13.68	522	18	70	986	126 176	16.10 13.00
15	3	13	4.3	5.5	10.70	286	95	55	1000	185	11.30
16	44	248	3.02	3.9	7.65	2562	58	40	1026	300 652	14.30 13.00
17	70	337	3.1	2.2	4.36	3166	45	20	909	380 516	14.70 12.60
18	11	12	1.1	0.6	1.20	49	4	4	—	—	—
19	—	—	—	—	—	—	—	—	—	—	—
20	—	—	—	—	—	—	—	—	—	—	—

\* Considerable losses.

Table I shows the results in general. These are the first figures to be published for the weight of the egg-white at different stages, and it is interesting to note how it disappears altogether by the 18th day. Quantitative expression is thus afforded for what originally was an observation of William Harvey [1653]. Col. 6 shows what percentage of the whole egg is taken up by the white, and is calculated, allowing for the loss of water during incubation, from

the figures of Murray [1925, Fig. 2]. It descends from 21 to 0 %. Col. 7 shows the ovomucoid isolated in g. and cols. 8 and 9 in g. per egg; it falls from 0.128 to 0.004. Although it is not claimed that there was a quantitative recovery of ovomucoid (and indeed these figures are much less than the fragmentary ones of Komori) yet, as the same care was used all through, it is probable that they have some relative significance. When they are compared with those of col. 5 and expressed as percentage of the wet weight of egg-white, as is done in col. 10, it is interesting to note that the amount of ovomucoid in 100 g. of wet egg-white remains constant throughout development at about 1 g. As far as these figures go, then, they confirm the observations of Bywaters and indicate that there is no preferential absorption of ovomucoid from the white, although there may be from the yolk. This latter possibility is made likely by the fact that the hump on the ovomucoid-glucose curve comes just between the two times at which the mucoprotein-glucose inside the embryo is high.

Col. 11 shows the mg. of ovomucoid taken each day for hydrolysis, and col. 12 the values found for the carbohydrate content of the molecule calculated as glucose. The average value is 11.5 %. Probably this represents the most accurate figure so far obtained, for all previous estimations were performed with copper methods and, as Holden [1926] has shown, in no conditions are these more unreliable than when dealing with protein hydrolysates. The values are tabulated as follows:

Investigator	% glucose in ovomucoid
Hofmeister [1898]	15.0
Seemann [1898]	29.4
Langstein [1900]	10.5
Willanen [1906]	19.5 to 22.3
Pavy [1907]	21.7
Samuely [1911]	34.9
Neuberg and Schewket [1912]	24.0
Zeller [1913]	33.7
Average	23.4
Present results	11.5

The most striking thing about col. 12, however, is that when the figures are plotted on a graph, an unmistakable upward trend is seen. The line which is drawn, having regard to all the points, traverses the graph, rising all the time from 7.4 on the 5th day to 15.4 on the 18th. No explanation has been found for this phenomenon. It is at any rate clear that there is no marked increase in the glucose content of ovomucoid at the period when it would be expected in the former of the two views outlined above. The mucoprotein of the yolk is therefore probably much increased at that time, and this will henceforth be used as a working hypothesis.

The gradual trend of the ovomucoid toward a higher glucose content may be regarded as one of those slow intramolecular changes which take place during embryonic development, a good example of which has recently been afforded by Plimmer and Lowndes [1927] in their work on the amino-acid distribution in the proteins of the hen's egg before and after incubation.



## II. THE EXISTENCE AND LOCALISATION OF OVOMUCOIDASE IN THE HEN'S EGG.

In 1899 Müller and Masayama [1899, 1900] reported the discovery of an active enzyme in the yolk of unincubated hens' eggs which would hydrolyse starch. This diastase was destroyed by heat and "under favourable conditions" would convert 45 % of a 3 % starch solution into the soluble forms of dextrin and isomaltose in 24 hours at 37°. This put on a sure basis the earlier and rather doubtful results of Krukenberg [1879] and was in turn confirmed by Diamare [1909], Herlitzka [1906] and Roger [1908]. Idzumi [1924] brought forward data which showed that the activity of the extra-embryonic diastase markedly increased as development proceeded, especially after the 15th day (rising from 40 to 640 units). Finally, Komori [1926] demonstrated that most if not all of the glucose in the ovomucoid molecule could be liberated by incubation with diastase prepared from meal. It remained to demonstrate that the egg itself, or certain parts of it, possessed the power of hydrolysing ovomucoid.

Experiments with this aim in view were carried out as follows. About fifty eggs were incubated under standard conditions for 5 days. At the end of that time they were broken open and divided into fractions thus: (a) the embryonic body, (b) the blastoderm and yolk-sac, (c) the egg-white, and (d) the yolk. The embryos themselves were placed one by one in distilled water in a small crystallising vessel under a strong beam of light and carefully freed from their amniotic membrane and the small allantois. After having been well washed in distilled water they were ground up with clean quartz sand in a mortar. The blastoderms and yolk-sacs were collected by pouring yolks and whites together into a large volume of water from which the membranes could with care be fished out. They were washed for 6 hours in a strong stream of water and finally ground up with quartz sand. The yolk and white were collected from other eggs with as little contamination as possible and were placed on a shaking machine for 2 hours with broken porcelain in order to mix them well. Then into a series of conical flasks were pipetted 50 cc. each of a solution of pure 5th day ovomucoid previously prepared and so arranged that each trial should contain 400 mg. of ovomucoid. Into a parallel series of flasks were run 50 cc. each of water, and to one of each series were added 50 cc. of embryo extract, yolk, etc., as the case might be. Toluene was added to 10 %. The flasks were plugged with cotton wool, and incubated at 37° for 3 days exactly, being shaken once a day. In view of the preliminary nature of these experiments, the  $p_{H}$  was not controlled by buffers. Glucose liberated was estimated in all the samples by the Hagedorn-Jensen method following precipitation with phosphotungstic acid. Table II gives the results.

As can be seen from Exp. K the ovomucoid preparation itself contained no free glucose at all, but when incubated alone about 2 mg. were split off, perhaps because of the presence of minimal amounts of the enzyme. This auto-digestion effect must be subtracted from the apparent results of col. 6 and the corrected values are shown in col. 7. It will be noted in the first place that the

Table II. *Experiments on ovomucoidase.*

1	2	3	4	5	6	7	8	9	10	11
Exp.	Fraction tested	Substrate added	3 days' incubation at 37°	mg. glucose found	Difference due to ovomucoid hydrolysis	Corrected for Exp. L	Yield % of theoretical	Dry wt. of sample g.	Col. 7 as % dry wt.	pH of system during incubation
A	Embryo extract	-	+	5.83	0.49	0.00	0.00	0.351	0	7.0
B		+	+	6.32						
C	White	-	+	5.04	2.31	0.44	1.49	6.00	7.3	8.1
D		+	+	7.35						
E	Yolk	-	+	17.70	11.10	9.23	31.20	15.00	61.6	5.4
F		+	+	28.80						
G	Yolk-sac and blastoderm	-	+	0.15	3.98	2.11	7.13	—	—	6.9
H		+	+	4.13						
K	Water	+	-	0.00	—	—	—	—	—	7.1
L		+	+	1.87						

slight excess of glucose in the embryo preparations is much more than accounted for by the correction of Exp. L, the effect of the egg-white is much reduced, but the yolk and the yolk-sac results still retain considerable magnitude. The conclusion, therefore, is that the enzyme ovomucoidase is not contained in the embryonic tissues themselves and only to a negligible extent in the egg-white, but that the yolk is very rich in it and that the blastoderm and yolk-sac also show a fair activity. Thus the embryo tests yield 0 % of the theoretical, the white 1.49, the yolk 31.2 and the yolk-sac 7.13. This conclusion is not affected by the varying amounts of solid material which must have been contained in the original samples, for as cols. 9 and 10 show, the yolk even so retains its superiority.

These results fit in well with what is already known. Roger, for instance, found more diastatic activity in the yolk than in the white. Bywaters [1913], by comparing the ratio

$$\frac{\text{g. uncoag. N per 100 g. egg-white}}{\text{g. glucose per 100 g. egg-white}},$$

came to the conclusion that the ovomucoid of the egg-white was not split up into protein and sugar moieties before absorption; but he only investigated the white, and for that fraction alone his results do not conflict with those now brought forward. The ovomucoid must be pictured passing from the white to the yolk, and there being split up into free glucose and protein before absorption into the embryo through the vitelline veins. It is interesting that the embryo possesses no ovomucoidase or very little; the hydrolysis must therefore be considered to take place outside it. The passage of ovomucoid from white to yolk is not difficult to imagine, for the existence of a current of water and of free glucose yolkwards has been clearly demonstrated [Aggazzotti, 1919; Needham, 1927]. Idzumi's observations on the increase of activity of the yolk diastase during development fit in perfectly with the increasingly rapid

disappearance of ovomucoid seen in Table I, col. 9. It would be very desirable to extend these observations by investigating the effect of  $p_H$ , salt concentration, etc., on the enzyme and by determining whether its activity remains the same in all fractions during development.

#### SUMMARY.

1. The carbohydrate content of the ovomucoid molecule, calculated as glucose, appears to be on an average 11.5 %. But there is a definite trend upwards as development proceeds, only 7.4 % being present on the fifth day but as much as 15.4 % on the 18th day.

2. An enzyme which hydrolyses ovomucoid appears to exist in the yolk and the yolk-sac of the hen's egg at the 5th day of development, possibly also in the white and the blastoderm, but not in the embryo.

3. Utilisation of ovomucoid by the embryo probably consists in a regular passage of ovomucoid into the yolk, where it is hydrolysed before being absorbed through the vitelline veins at a rate which varies during the incubation period.

The writer wishes to thank Sir F. G. Hopkins for his constant interest and the Government Grant Committee of the Royal Society which provided a grant covering the cost of these researches.

#### REFERENCES.

- Aggazzott (1919). *Arch. Sci. Biol.* **1**, 120.  
 Bywaters (1913). *Biochem. Z.* **55**, 245.  
 Diamare (1909). *Rend. Acc. Fis. Math. Nap.* **15** (3), 319.  
 Hagedorn and Jensen (1923). *Biochem. Z.* **135**, 48.  
 Harvey (1653). *De Generatione Animalium*, Exercit. xxxvi, p. 183.  
 Herlitzka (1906). *Biologica*, **1**, 80.  
 Hofmeister (1898). *Z. physiol. Chem.* **24**, 159.  
 Holden (1926). *Biochem. J.* **20**, 263.  
 Idzumi (1924). *Mitt. Med. Fak. Tokyo*, **32**, 197.  
 Komori (1926). *Jap. J. Biochem.* **6**, 1.  
 Krukenberg (1879). *Unters. physiol. Inst. Heidelberg*, **2**, 273.  
 Langstein (1900). *Z. physiol. Chem.* **31**, 49.  
 Müller and Masayama (1899). *Sitz. Phys.-Med. Ges. Würzburg*, p. 95.  
 ——— (1900). *Z. Biol.* **39**, 547.  
 Murray (1925). *J. Gen. Physiol.* **9**, 1.  
 Needham (1926). *Brit. J. Exp. Biol.* **4**, 114.  
 ——— (1927). *Brit. J. Exp. Biol.* (In the press.)  
 Neuberg and Schewket (1912). *Biochem. Z.* **44**, 491.  
 Pavy (1907). *Carbohydrate Metabolism*, p. 44.  
 Plimmer and Lowndes (1927). *Biochem. J.* **21**, 254.  
 Roger (1908). *J. Physiol. Path. Gen.* **10**, 797.  
 Samuely (1911). *Biochem. Handlex.* **4**, 147.  
 Seemann (1898). *Dissert. Marburg*.  
 Willanen (1906). *Biochem. Z.* **1**, 109.  
 Zeller (1913). *Z. physiol. Chem.* **86**, 85.

# XCIX. A QUANTITATIVE STUDY OF SUCCINIC ACID IN MUSCLE. II.

## THE METABOLIC RELATIONSHIPS OF SUCCINIC, MALIC AND FUMARIC ACIDS.

By DOROTHY MOYLE NEEDHAM (*Beit Memorial Research Fellow*).

*From the Biochemical Laboratory, Cambridge.*

(Received April 30th, 1927.)

### INTRODUCTION.

THAT the succinic acid content of fresh, chopped muscle tissue increases on incubation in buffer solutions of suitable  $p_H$  has been shown in a previous paper [Moyle, 1924]. It would obviously be of the greatest assistance towards elucidating the rôle of the succinic acid in the intact muscle if one could discover whether the succinic acid maximum reached *in vitro* is an equilibrium point or not; that is to say, whether the formation of more succinic acid is hindered by the accumulation of the acid. If this were the case, we might expect that, after removal of the accumulated acid by admission of oxygen, putting the system back under anaerobic conditions would lead to a renewal of the maximum from some unknown precursor.

When such experiments were performed, it was indeed found that after a period in nitrogen, followed by a period in oxygen involving decrease in succinic acid content, a return to nitrogen was accompanied by rise in concentration of this acid.

There is of course an alternative explanation to the one just put forward of the renewed succinic acid maxima: that the fumaric and malic acids formed in presence of oxygen [Batelli and Stern, 1911, 1; Einbeck, 1919] are themselves not oxidised or only very slowly; when anaerobic conditions again prevail these acids might be reconverted into succinic acid. As regards the possibility of the removal of fumaric and malic acids by oxidation, Batelli and Stern [1911, 2] showed that these acids, as well as citric acid, were oxidised to some extent by chopped muscle suspended in three times its weight of water, though the oxygen uptake is very much less rapid than in the oxidation of succinic to fumaric acid. Thorough washing they found to destroy the power of the tissues to bring about the former oxidations. Other workers [Thunberg, 1909, 1911, 1 and 2; Meyerhof, 1919; Grönvall, 1924] have observed increased oxygen uptake upon adding fumaric acid to muscle after various degrees of extraction. The oxidation of all these acids is inhibited by the presence of

inorganic salts [Batelli and Stern, 1911, 2], but with the fumaric and malic acids, if we may judge from the figures given for citric acid, not more than with the succinic acid.

When the alternate rise and fall in succinic acid content had been established, it therefore became necessary to determine the amounts of fumaric and malic acids present at the same times, and a method is described for estimating the malic acid, as well as the total quantity of the three acids together, in the same muscle sample. From these two values, as is explained later, a rough calculation may be made of the succinic and fumaric acids present. Using these methods, the experiments with alternate atmospheres were repeated, and it was found that the total content of the three acids does not remain constant, but rises and falls markedly with the succinic acid content. Simple interconversion of succinic acid on the one hand and the oxidation products malic and fumaric acids on the other, is not then the explanation of the results obtained.

#### THE EFFECT OF ALTERNATE AEROBIC AND ANAEROBIC CONDITIONS ON THE SUCCINIC ACID CONTENT.

The first result which emerged from the following experiments was the difficulty of obtaining complete disappearance of the succinic acid in oxygen, although, as is well known, succinic acid added to washed muscle is rapidly and quantitatively converted into fumaric acid [Fleisch, 1924].

Muscle removed from the neck of the bullock immediately after slaughtering was brought to the laboratory in a pan surrounded by a freezing mixture; a long, cylindrical muscle could be dissected out with little injury, and this, after further cooling, was minced and samples were weighed out. After the required time of incubation the flask was cooled, and an equal volume of alcohol was added (counting each g. of muscle as 1 cc. in the suspension). The succinic acid was estimated according to the method previously described.

*Exp. 1.* Four 50 g. lots were used, each suspended in 100 cc. of buffer solution at  $p_H$  7.4. Two of the flasks also contained about 50 mg. each of succinic acid. One of these flasks and one control were connected to a nitrogen cylinder, the remaining two to an oxygen cylinder. All were left at 33° for 1.5 hours.

					Succinic acid in mg. per 50 g.
Control in oxygen	...	...	...	...	8.5
"	+ succinic acid in oxygen	...	...	...	28.8
Control in nitrogen	...	...	...	...	12.8
"	+ succinic acid in nitrogen	...	...	...	54.4

It seemed possible that the conditions might be unfavourable for complete oxidation, either of the succinic acid to fumaric, or of the fumaric acid further (with consequent retardation of fumaric acid formation) owing to the inhibitory effect of the phosphate in the buffer and of too low a  $p_H$  (according to Ohlsson the optimum  $p_H$  is about 8.7). A buffer solution much less concen-

trated was therefore made up, following the directions given by Ohlsson [1921] for the buffer that he used with his enzyme preparation. The  $p_H$  of this solution was 9.0, and it was ascertained by means of the hydrogen electrode that the addition of 40 g. of beef to 500 cc. did not cause the value to fall below 7.47, even after several hours.

*Exp. 2.* 40 g. lots of beef were used, each suspended in 500 cc. of the dilute buffer; 50 mg. of succinic acid were added to each bottle and both were oxygenated at 37°.

	Succinic acid in mg. per 40 g.
After 40 mins.	28.2
After 60 mins.	28.8

It is clear that these precautions have not overcome the difficulty of incomplete removal, which must be due to some other factor than the effects of the medium already considered. For instance, it is possible that under the conditions of the experiment continuous production of succinic acid is taking place, while with washed muscle the precursor has been removed. Further attempts, therefore, to obtain complete disappearance were abandoned, and the experiments on anaerobiosis and oxygenation were begun.

In the remaining experiments described in this paper a somewhat different arrangement was used; the desired atmosphere was obtained by evacuating the flasks till the contents boiled, and then filling with the required gas until a little more than atmospheric pressure was reached. As it had been found that pigeon breast muscle gave a higher yield of succinic acid than any other kind tried, this muscle was used; four pigeons were generally taken for each experiment. After killing, bleeding and plucking, they were placed breast downwards on ice for a short time; then the two breast muscles were dissected off from each with as little injury as possible, further cooled in a covered glass dish packed in freezing mixture, and minced. As the succinic acid content varies considerably from one pigeon to another, very thorough mixing was essential before samples were weighed out. The anaerobic flasks were always evacuated before removal from the bath, and were opened with a side-tube dipping under alcohol, so that the contents were precipitated without admission of air. The oxygen flasks were shaken almost continuously.

*Exp. 3.* Temp. 33°. Phosphate buffer,  $p_H$  9.0 (1.4 %  $Na_2HPO_4 \cdot 2H_2O$ ); two volumes used.

1.0 p.m.	All flasks filled with nitrogen.
2.0 "	First flask, N 1, removed; oxygen introduced into remaining four.
2.40 "	Second flask, O 1, removed; nitrogen introduced into the others.
3.20 "	Third flask, N 2, removed; oxygen introduced into remaining two flasks.
4.0 "	Fourth flask, O 2, removed; nitrogen introduced into the last flask.
5.15 "	Last flask, N 3, removed.

The results are shown in Fig. 1.

*Exp. 4.* Temp. 33°. The concentration of phosphate was halved and the volume used was doubled. All the periods were shortened (see Fig. 1). The results were very like those in Exp. 3.

*Exp. 5.* This experiment was similar to the last, except that at the end of

the first nitrogen period all the flasks but N 1 were opened and 20 cc. of 0.2 *N* sodium hydroxide were added to each. It was calculated that this should raise the  $p_H$  by about one unit, and the final  $p_H$  was found to be about 7.3 instead of below 7.0, as in the previous experiment. This change in the treatment does not seem to affect the results (Fig. 1).

The combined results of these three experiments seem to show that, as time goes on, the possibility of the complete removal of succinic acid in oxygen becomes progressively greater. When the second oxygen period falls, 3 hours after the beginning of the experiment, disappearance is almost complete. Renewed maxima in nitrogen are obtained, but the value in each is generally somewhat lower than in the previous one.

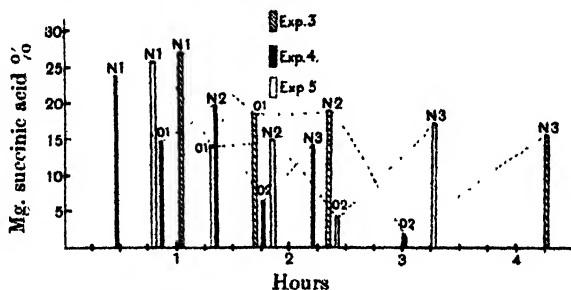


Fig. 1.

#### THE METHOD OF ESTIMATION OF MALIC ACID AND OF THE THREE ACIDS TOGETHER.

In the method used for the estimation of the total quantity of the three acids, they were quantitatively precipitated as the silver salts by adding  $\text{AgNO}_3$  to a neutral solution in 36 % alcohol; the silver was then determined by titration with *N*/100 KCNS. After removal of the silver precipitate, the malic acid was estimated polarimetrically as the molybdenum compound, which, as is well known, shows an enormously increased rotation.

As two important difficulties were encountered in the working out of the method, it might be well to state the reason for some of the processes described.

(1) It was desirable to extract with ether before the silver precipitation to get rid of traces of amino-acids, purines, etc., and also the greater part of the phosphate (if the whole of the phosphate of the buffer is removed with barium, there is much loss by adsorption of the acids, especially succinic, on the barium phosphate precipitate). On account of the high partition coefficient of malic acid between water and ether (62 : 1) it was necessary to put the solution on to some water absorbent, and to extract this in a Soxhlet. As water absorbents, plaster of Paris, anhydrous sodium sulphate and fat-free filter paper strips were tried, but found useless because the malic acid, once adsorbed, could not be quantitatively removed. Only about 66 %, and sometimes much less, could be recovered when the pure acid was tested. In the presence of high concentrations of salts, *e.g.* ammonium sulphate, in the liquid adsorbed, the

recovery was much less; and when a muscle extract with added malic acid was tried, none of the acid could be recovered. Using silica gel<sup>1</sup>, on the other hand, 90 % of the acid could be found again after 6 hours' extraction, when the pure acid was tried; and 84 % was recovered after 24 hours' extraction in the presence of phosphate and lactate in amounts comparable to those in a muscle sample. No explanation of these phenomena inhibitory to extraction can be offered; in the case of the lactate some indications were obtained that, even in the presence of silica gel, the malic acid only begins to be extracted when all or most of the lactic acid has been extracted, and for this reason the time of extraction was made lengthy.

(2) In the presence of so large a proportion of lactic acid, it was found that the usual precipitation methods for malic and succinic acids, especially the former, fail. Thus for succinic acid alone very good results were obtained for 10 mg. of the acid by precipitating the calcium salt in 87 % alcohol, and, after filtering and slight washing, dissolving the precipitate in water and estimating as the silver salt; but on addition of 150 mg. lactic acid, practically no precipitate was obtained on adding alcohol. Using the barium salts, in presence of 300 mg. lactic acid, 10 mg. succinic acid was precipitated to the extent of 70 % only. 300 mg. lactic acid almost entirely prevents the precipitation of 10 mg. malic acid whether as the calcium or the barium salt in 85 % alcohol.

Similar results were obtained for the silver methods. Precipitation in 36 % alcohol solution (3 cc. alcohol to 5 cc. water) had been adopted because, in this medium, at  $p_H$  6.2-7.2 the three acids are quantitatively precipitated as the silver salts, whilst in aqueous solution only about 60 % of the malic acid is precipitated. When lactic acid was present, however, to the extent of 40 times the malic acid, only about 50 % of the latter was precipitated; the succinic acid precipitation was less affected. In order to overcome this difficulty, the practice was adopted of a preliminary silver precipitation in 75 % alcohol; in this way practically all the lactic acid is removed, and the precipitate obtained, which gives a high percentage of silver and probably contains basic salts, is dissolved and re-precipitated as described later. This double precipitation is unnecessary if the proportion of lactic to malic acid is not greater than 20 : 1, but, as we shall see, it is usually greater than this in muscle.

Complete removal of the lactic acid is also necessary for the polarimetric estimation of malic acid, as the rotation of the former acid is also markedly increased on addition of molybdenum.

The details of the method finally used were as follows. The 50 % alcoholic extract is strained from the muscle residue by pressing through muslin, and the muscle washed twice with 50 % alcohol. The extract is filtered through paper, neutralised, and evaporated *in vacuo* at 40°. When the volume is reduced to about 75 cc., the precipitated protein is filtered off and the filtrate

<sup>1</sup> The commercial name of the product used was "Super-Cel Hyflo," kindly supplied to me by the makers, the Celite Products Corporation.



evaporated on the water-bath to about 7 cc.; it is cooled on ice and 2 cc. of 50 %  $\text{H}_2\text{SO}_4$  (by volume) added. The acid extract is now adsorbed on a silica gel, the dry powder is well mixed and ground, and placed in a Soxhlet thimble. After 1 hour's extraction with light petroleum to remove most of the fat, it is extracted for about 90 hours with ether.

When the extraction is finished, a little water is added, and the ether is distilled off. The warm aqueous solution is neutralised with saturated baryta solution to remove sulphate and phosphate. The neutralised solution should not be allowed to stand very long or remain very hot before filtration, on account of the risk of converting into a less soluble form the barium salts of the acids to be estimated. The barium precipitate is filtered off and well washed with hot water.

The filtrate is evaporated down to 10 cc., and 30 cc. 97 % alcohol are added. The alcoholic solution is adjusted to  $p_{\text{H}}$  7.0 (using phenol red) and 1-2 cc. 10 %  $\text{AgNO}_3$  solution is added. The silver precipitate contains all the succinic, malic and fumaric acids, but very little lactic acid. The precipitate is filtered off in a Gooch crucible, washed with 75 % alcohol, suspended in water with a few drops of 1 % sulphuric acid and treated with hydrogen sulphide. After removal of the silver sulphide by filtration and of the hydrogen sulphide by aeration, the solution is neutralised with sodium hydroxide and evaporated down to 20 cc. 12 cc. 97 % alcohol are now added, the solution is brought to  $p_{\text{H}}$  7.0, and 10 %  $\text{AgNO}_3$  is again added. The silver salts of the succinic, malic and fumaric acids are now quantitatively precipitated; in order to estimate their amount, the precipitate is ground with warm water containing a few drops of 50 %  $\text{H}_2\text{SO}_4$ , and the cold solution is titrated with 0.01N KCNS, using iron alum in nitric acid as outside indicator.

The silver thiocyanate is filtered off, and in the filtrate the malic acid is estimated polarimetrically as the molybdenum compound, according to the method of Auerbach and Kruger [1923]. The filtrate is neutralised and evaporated down to 8.4 cc., and its rotation taken in a 2 dm. tube. After concentration to 4 cc., 4 cc. of 14.2 % ammonium molybdate solution are added, and 0.4 cc. of glacial acetic acid; when the solution has stood in the dark for 2 to 3 hours, the increase in rotation is determined. Using the mercury green line, it was ascertained that 1 mg. of malic acid gave, under these conditions, a rotation of  $+0.21^\circ$ .

The method was tested by adding known amounts of the acids to muscle, suspended in two volumes of 1.4 %  $\text{Na}_2\text{HPO}_4$ ,  $2\text{H}_2\text{O}$ , and some results, after subtracting the small quantities found in the control, are shown in Table I.

These figures have been arranged in a diagram (Fig. 2), and it will be seen that, with quantities of malic acid between 45 mg. and 8 mg. added to about 50 g. muscle, the amount recoverable falls off gradually from about 80 % to about 55 %; below 8 mg. only about 35 % is recoverable. The points marked X were obtained by the double precipitation method, which gives better results in these regions of low concentration. The low results obtained even

with this method for amounts below 8 mg. cannot be due to the effect of lactic acid, but are probably brought about by adsorption of the barium malate on the barium phosphate precipitate.

Table I.

Muscle used	Amount added	Amount found	% recovered
40 g. beef	43 mg. malic acid	34.0 mg.	79
40 "	21.5 "	13.1	61
40 "	10.75 "	5.8	54
45 "	30 "	24.0	80
45 "	50 "	37.5	75
45 "	10 "	3.7	37
50 "	10 "	3.4	34
50 g. pigeon	8.5 "	5.3	62
50 "	5.6 "	1.8	32
50 "	3.8 "	1.2	32
45 g. beef	30 mg. succinic + 30 mg. fumaric	42.0	70
45 "	" " " "	42.0	70
50 g. pigeon	21.7 mg. succinic + 3.9 mg. fumaric	21.0	82
50 "	" " " "	15.4	60
50 "	" " " "	17.0	66

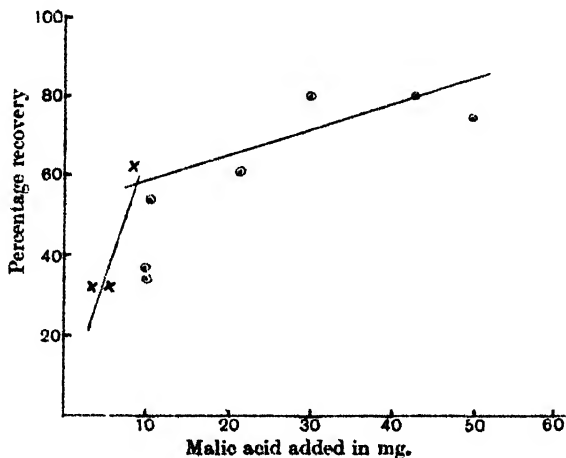


Fig. 2.

In order to test whether the silver precipitate obtained consisted only of succinate, malate and fumarate (one or more of these acids) the silver in weighed amounts was estimated.

(a) The silver precipitate obtained in nitrogen weighed 58.7 mg. and contained 65.1 % of silver; the calculated percentage, as the precipitate was found to contain 3.4 mg. of silver malate, is 64.7.

(b) The silver precipitate obtained from an initial sample weighed 54.5 mg., and this contained 64.1 % of silver. Allowing for the 5.7 mg. of silver malate contained in it, the calculated percentage was 64.5.

In this method the removal of hydroxy-acids with acid permanganate is avoided; there is therefore no risk, as in the previous method for succinic acid, of the breakdown of possible traces of 5-carbon chain acids to succinic acid in the course of the estimation.

THE EFFECT OF AEROBIC AND ANAEROBIC CONDITIONS ON THE MALIC ACID CONTENT AND ON THE TOTAL CONTENT OF THE THREE ACIDS.

Pigeon breast muscle was used in these experiments, and the arrangement was similar to that in Exps. 3, 4 and 5. As will be seen, the malic acid found per 50 g. muscle always lies below 8 mg., that is, in the region of only about 35 % recovery; in order, therefore, to compare the total amount of acids under the different conditions, this amount of malic acid is doubled, and thus can be compared with the amounts of succinic and fumaric acids found. Of the latter acids, as we have seen, about 70 % is recovered. The fumaric acid is reckoned as present to the extent of 50 % of the malic (according to Einbeck [1919] and Dakin [1922] this is roughly the ratio found at equilibrium when the acids are in the presence of the muscle enzyme). The succinic acid is found by difference.

*Exp. 6.* Two samples were ground immediately after removal from the animal with a mixture of equal volumes of ice-cold alcohol and ice-cold 0.7 %  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ; three other samples remained at 33° in four volumes of 0.7 %  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , one in nitrogen for 3 hours, the second in oxygen for 1 hour and the third in oxygen for 3 hours.

	Malic acid in mg. per 100 g.	
	Found	Corrected
Initial (1)	9.7	19.4
" (2)	12.0	24.0
In nitrogen	1	2
In oxygen 1 hour	1.5	3
" 3 hours	1	2

*Exp. 7.* Temp. 37-39°; each muscle sample in two volumes of 1.4 %  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ .

11.45 a.m.	Flasks filled with nitrogen.
1.45 p.m.	N 1 removed; other flasks filled with oxygen.
2.45 "	O 1 removed; remaining flask filled with nitrogen.
3.45 "	N 2 removed.

In order to inhibit if possible the oxidation of malic acid whilst not interfering with the oxidation of succinic acid to malic acid, the addition of arsenic was tried; according to Batelli and Stern the latter oxidation is not markedly slowed until the concentration of arsenious acid reaches 1 : 1000, while the further oxidation of malic acid is inhibited at a concentration of 1 : 10,000. A fourth flask was therefore exhausted at 12.0 noon and filled with nitrogen; at 2.0 p.m. 15 mg. of arsenious acid (dissolved in a little 0.1 *N* sodium hydroxide) were added, and the atmosphere was changed to oxygen. The flask was repeatedly shaken till 3.0 p.m., when alcohol was added.

An initial sample, precipitated at once with alcohol and an equal volume of phosphate solution, was also worked up.

# SUCCINIC, MALIC AND FUMARIC ACIDS IN MUSCLE 747

	Malic acid found in mg. %	Malic acid corrected in mg. %	Total silver in cc. KCNS %	Amounts of acids calculated in Ag ppt. in mg. %	Total acid with malic corrected in mg. %
Initial	6.3	12.6	37	6.3 malic 6.3 fumaric 12.2 succinic	31.1
N 1	1	2	80	1.0 malic 1.0 fumaric 47.8 succinic	50.8
O 1	2.4	4.8	40	2.4 malic 2.4 fumaric 20.6 succinic	27.8
N 2	Nil	Nil	72	44.6 succinic	44.6
With arsenic	2.0	4.0	32	2.0 malic 2.0 fumaric 16.4 succinic	22.4

*Exp. 8.* This experiment was like the previous one, except that hydrogen, passed slowly through two pyrogallol bottles, was used for obtaining the anaerobiosis. Temp. 35°; three 60 g. lots, each suspended in 130 cc. 1.4 %  $\text{Na}_2\text{HPO}_4$ ,  $2\text{H}_2\text{O}$ . An initial sample was also worked up.

1.10 p.m. Flasks filled with hydrogen.  
 3.10 „ H 1 removed; oxygen introduced.  
 4.0 „ O 1 removed; hydrogen introduced.  
 4.45 „ H 2 removed.

	Malic acid found in mg. %	Malic acid corrected in mg. %	Total silver in cc. KCNS %	Amounts of acids calculated in Ag ppt. in mg. %	Total acid with malic corrected in mg. %
Initial	9.3	18.6	57.3	9.3 malic 9.3 fumaric 18.0 succinic	46
H 1	1	2	77	1.0 malic 1.0 fumaric 46.0 succinic	49
O 1	2	4	25	2.0 malic 2.0 fumaric 11.8 succinic	17.8
H 2	1.2	2.4	53	1.2 malic 1.2 fumaric 30.7 succinic	34.3

*Exp. 9.* In this experiment the method of double silver precipitation was used; in other details the procedure was the same as for Exp. 8, except that an extra hydrogen and an extra oxygen period were introduced.

1.0 p.m. Flasks filled with hydrogen.  
 3.0 „ H 1 removed; oxygen introduced.  
 3.35 „ O 1 removed; hydrogen introduced.  
 4.10 „ H 2 removed; oxygen introduced.  
 4.45 „ O 2 removed; hydrogen introduced.  
 5.20 „ H 3 removed.

Initial	Malic acid found in mg. % 4	Malic acid corrected in mg. % 8	Total silver in cc. KCNS % 41.4	Amounts of acids calculated in Ag ppt. in mg. %	Total acid with malic corrected in mg. %
				4.0 malic 4.0 fumaric 19.4 succinic	31.4
H 1	1	2	61	1.0 malic 1.0 fumaric 37.8 succinic	40.8
O 1	—	—	6	3.9 succinic	3.9
H 2	1	2	38	1.0 malic 1.0 fumaric 22.9 succinic	25.9
O 2	—	—	4	2.6 as succinic	2.6
H 3	—	—	12.4	8.1 as succinic	8.1

*Exp. 10.* The power of the minced muscle was tested as regards the oxidation of added succinic and malic acids in the later stages of incubation.

Three samples were kept in hydrogen for 2 hours; then one was removed and precipitated. Oxygen was introduced into the other two, and to one a solution containing 25.5 mg. of succinic acid and 25 mg. of malic acid (neutralised with sodium hydroxide) was added; both flasks were kept in the bath for an hour. Temp. 35°; two volumes of 1.4 %  $\text{Na}_2\text{HPO}_4$ ,  $2\text{H}_2\text{O}$  used.

	Malic acid found in mg. per 50 g. 1.3	Malic acid corrected 2.6	Total silver in cc. KCNS per 50 g. 32.1	Amounts of acids calculated in Ag ppt. mg. per 50 g.	Total acid with malic corrected in mg. per 50 g.
H 1				1.3 malic 1.3 fumaric 18.5 succinic	22.4
O 1	0.2	0.4	3.0	0.2 malic 0.2 fumaric 1.65 succinic	2.25
O + 25 mg. malic + 25.5 mg. succinic	5.8	11.6	20	5.8 malic 5.8 fumaric 2.1 succinic	19.5

### DISCUSSION.

Perhaps the most important result of this work has been the evidence gained of the removal in oxygen of part of the succinic acid (probably by transformation into carbon dioxide and water, or at any rate into a substance not precipitated by silver in 36 % alcohol) and the renewed formation of the acid in the absence of oxygen. The explanation which may be put forward for the present is that accumulation of the acid up to a certain point hinders further formation; possibly there is a back reaction according to the mass action law. In oxygen when the accumulation is removed renewed formation takes place, and *in vivo* we may suppose that a continuous production and removal go on. As to the nature of the precursor in the muscle, there is at present no information.

Two suggestions may be made as to the cause of the greater removal in oxygen of the three acids at later stages in incubation. One is that oxidation is in some way facilitated; the other that in the early stages production of the acids is more vigorous, falling off later. The latter explanation seems the more

likely. There is no reason to suppose that production ceases in oxygen, and that its activity falls off as time goes on is indicated by the progressively lower maxima on return to nitrogen. Further, according to Batelli and Stern [1911, 2], the power of the muscle to oxidise added malic and fumaric acids, far from becoming greater in course of time, falls off rapidly after 1 or 2 hours. In one experiment in the present work, out of 40 mg. added to 50 g. of muscle at the beginning of the third hour, only 21 mg. had been oxidised away by the end of the hour; the muscle therefore is very near the limits of its oxidising capacity, and the fact that the maximum in nitrogen can be practically completely removed in oxygen at this stage must not be taken as a sign that the muscle can do much more than this. It would be interesting to test the degree of oxidation of quantities of acid added at different times throughout incubation.

When this work was begun, nothing was known of the concentration of malic and fumaric acids occurring in muscle; the amounts of succinic acid under varying conditions had been ascertained [Moyle, 1924], and it was known from the work of Einbeck and of Dakin that, in the presence of the muscle enzyme, the equilibrium between fumaric and malic acids lay at about 70 % of the latter. But the equilibrium concentration under various conditions of succinic acid and its two oxidation products had never been investigated. It was hoped that possibly conditions might be found favourable to the oxidation of succinic acid to malic and fumaric acids, but unfavourable to the oxidation of the latter acids; in this way, by accumulation of these oxidation products, evidence might be obtained of succinic acid metabolism. In spite of the probability, however, that the oxidation of malic and fumaric acids falls off considerably an hour or two after the death of the animal, while the oxidation of added succinic acid can certainly go on vigorously for many hours, it has not so far been possible to obtain any accumulation of malic acid under aerobic conditions. Even the use of arsenious acid had no effect. This is probably because production of the acids falls off.

The disappearance, when the muscle is transferred to nitrogen, of the malic acid initially present shows that the change from succinic acid to malic is a reversible one. This was to be expected from the experiments of Thunberg [1925], of Batelli and Stern [1921] and of Ahlgren [1925] with added malic acid in the presence of methylene blue or of thionine.

The comparatively high concentration of malic acid in the fresh muscle is interesting; probably the more *post mortem* change were avoided, the higher would be the concentration found, as during the necessary manipulation the muscle suffers oxygen deprivation. In this connection, it is interesting to notice that Einbeck [1914] prepared fumaric acid from fresh beef; he obtained a yield of 2.8 mg. per 100 g. of muscle, but considerably more was probably present, as the method he used of silver precipitation in acid solution is not quantitative. The concentration of malic acid in the oxygenated samples never reaches that found in the initial sample; probably the conditions in the living muscle for production and oxidation of the acids cannot be, or have not yet been, successfully imitated *in vitro*.

## SUMMARY.

1. A method has been worked out for the estimation of malic acid in muscle, and of the total amount of succinic, fumaric and malic acids taken together.

2. When minced muscle, suspended in buffer solution, is placed alternately under anaerobic and aerobic conditions, the succinic acid content rises in the nitrogen, falls in the oxygen, rises again in the nitrogen, and so on.

3. With similarly repeated changes of atmosphere, the total amount of succinic, fumaric and malic acids taken together rises in anaerobiosis and falls on oxygenation.

4. The malic acid content is highest in the fresh muscle (about 12–18 mg. per 100 g.); it falls to practically nothing in nitrogen, and on admission of oxygen it may rise, but very slightly.

5. When succinic acid disappears in oxygen it is oxidised further than to fumaric and malic acids; and when the succinic acid maximum is renewed in nitrogen, the latter acid is formed from some other source than reversibly from fumaric and malic acids.

6. The results also suggest that production of succinic acid is hindered by accumulation of the acid, and make possible the hypothesis that *in vivo* production and oxidation go on continuously.

I wish to express my sincere thanks to Sir F. G. Hopkins for his most kind interest and encouragement in this work.

## REFERENCES.

- Ahlgren (1925). *Skand. Arch. Physiol.* Suppl. p. 24.  
 Auerbach and Kruger (1923). *Z. Nahr. Genussm.* **46**, 97.  
 Batelli and Stern (1911, 1). *Biochem. Z.* **30**, 172.  
 ——— (1911, 2). *Biochem. Z.* **31**, 478.  
 ——— (1921). *Compt. Rend. Soc. Biol.* **84**, 305.  
 Dakin (1922). *J. Biol. Chem.* **52**, 183.  
 Einbeck (1914). *Z. physiol. Chem.* **90**, 301.  
 ——— (1919). *Biochem. Z.* **95**, 296.  
 Fleisch (1924). *Biochem. J.* **18**, 294.  
 Grönvall (1924). *Skand. Arch. Physiol.* **45**, 303.  
 Meyerhof (1919). *Pflüger's Arch.* **175**, 20.  
 Moyle (1924). *Biochem. J.* **18**, 351.  
 Ohlsson (1921). *Skand. Arch. Physiol.* **41**, 77.  
 Thunberg (1909). *Skand. Arch. Physiol.* **22**, 431.  
 ——— (1911, 1). *Skand. Arch. Physiol.* **24**, 23.  
 ——— (1911, 2). *Skand. Arch. Physiol.* **25**, 37.  
 ——— (1925). *Skand. Arch. Physiol.* **46**, 339.

# C. QUANTITATIVE STUDIES OF THE NITRO-PRUSSIDE REACTION IN NORMAL TISSUES AND TUMOURS.

BY ERNEST LAURENCE KENNAWAY AND IZRAEL HIEGER.

*From the Cancer Hospital Research Institute, London.*

*(Received May 2nd, 1927.)*

THE analyses described below provide material for a comparison of the glutathione content of normal tissues and of tumours. The investigation consists of two parts concerned respectively with (a) human tissues, normal, malignant, and non-malignant; and (b) the tissues of the fowl, and the Rous fowl sarcoma. For the sake of simplicity the extractable material giving the nitroprusside test is spoken of here as glutathione, though the possible presence of another SH compound is not excluded.

## HUMAN TISSUES.

*Technique.* All the organs and tumours named in Table I, with the exception of the placenta, were obtained from the operating theatres of the Cancer Hospital; the material was brought as quickly as possible to the laboratory, dissected and weighed while still warm, and treated immediately by Tunnicliffe's method [1925]. Every effort was made to avoid necrotic material. The tissue was minced with scissors, ground with sand and trichloroacetic acid; the mass was then filtered on a Büchner funnel, and the grinding and filtration were repeated twice. The three filtrates were united, centrifuged and titrated. To ascertain that the extraction of the soluble glutathione was practically complete, the solid material was then ground and filtered a fourth and a fifth time, and the small amount of glutathione present in these filtrates titrated. Finally, a solution of nitroprusside, ammonia and ammonium sulphate was poured over the residue on the funnel to demonstrate the presence of fixed SH groups. For the titration, 2 cc. saturated ammonium sulphate solution, 2 drops strong ammonia, and 5 drops of 1 % sodium nitroprusside solution, not more than 1 hour old, were placed in a series of test-tubes. N/100 iodine was run in until 5-10 drops of the titrated fluid transferred to one of these test-tubes produced no colour. The total amount thus removed was shown to be not more than 2 or 3 % of the whole titre. After the end-point for nitroprusside was reached, a few drops of 1 % soluble starch were added, and the titration continued.



In practically every case, a portion of each tissue analysed was reserved for the preparation of histological sections. This is of great importance, for obviously it is essential to attempt an estimate as accurate as possible of the amounts of non-cellular material, fibrotic or necrotic, which are present. Even when every care is taken, it is impossible to be sure that the small pieces taken for section are really fair samples of 10 or 15 g. of tissue. This is the weak point in all such investigations. But if no microscopic examination were made one might for example estimate the glutathione in a scirrhus and in a medullary cancer of the breast, and be unaware that the "cancer" was very largely non-cellular, consisting in the one case chiefly of fibrous, and in the other of necrotic, material containing very few living cancer cells.

The results obtained with human tissues are shown in Table I.

*Notes on the microscopic appearances of the tissues analysed.*

(1) *Rectum.* One of the most suitable specimens obtainable for a chemical comparison of normal and malignant tissues is the rectum when removed by abdomino-perineal excision for cancer. A considerable length of normal mucous membrane is available immediately adjacent to the malignant tumour which has developed from it. Five such specimens have been examined. When the innermost layers of any part of the stomach or intestine are dissected off in the ordinary way, microscopic examination shows that the material consists of (a) mucous membrane, (b) muscularis mucosae, (c) a variable amount of submucous layer, which is the plane along which the separation takes place. Of these various tissues, the epithelium of the mucous membrane alone is comparable to the cancer, which has developed from it. Examination of a number of sections suggests that, on an average, something like one-half of the material analysed consists of this epithelium. In the cancer, on the other hand, however carefully this may be dissected out, considerable areas of muscle, fibrous and necrotic tissue, comparatively very poor in cells, will be found in sections; in some of these sections the cancer may occupy one-tenth or less of the area. Hence, when the mucous membrane and the cancer thus prepared yield approximately the same amounts of glutathione it seems very probable that the cellular part of the cancer contains more of this compound than does the corresponding cellular part of the normal mucous membrane.

(2) *Breast.* In Case 6 the normal part of the breast consisted very largely of fibrous and adipose tissue, with very little mammary gland; the cancer, on the other hand, was very cellular, and contained a hundred times more glutathione per cent. than did the rest of the breast. This case illustrates very well the great difficulty of obtaining exactly comparable normal and malignant tissues. Cases 7 and 8 showed typical non-malignant tumours; the former, an intracystic papillary fibro-adenoma, was considerably more cellular than the latter, which was a fibro-adenoma of the ordinary type. The more cellular tumour gave a much higher figure for glutathione (85 mg.) than the latter

Table I. *Human tissues.*

		mg. glutathione per 100 g. fresh tissue			Tumours	
		Normal			Innocent	Malignant
<i>Rectum</i>						
Case 1	Mucous membrane above tumour	48			—	Columnar-celled cancer
" 2	" " below	35			—	—
" 3	" " above	56			—	Columnar-celled cancer
" 4	" " below	47			—	—
" 5	" " above	50			—	Columnar-celled cancer
" 6	" " below	55			—	—
" 7	" " above	41			—	Columnar-celled cancer
" 8	" " below	50			—	—
" 9	" " above	52			—	Columnar-celled cancer
" 10	" " below	45			—	—
<i>Breast</i>						
Case 6	Breast tissue	0.9			—	Spheroidal-celled cancer
" 7	" "				Papilloma	—
" 8	" "				Fibro-adenoma	—
" 9	" "				—	—
" 10	" "				—	Spheroidal-celled cancer, primary
" 11	" "				—	Spheroidal-celled cancer, metastases, axillary glands
" 12	" "				—	Spheroidal-celled cancer, primary
" 13	" "				—	Spheroidal-celled cancer, primary
" 14	" "				—	Spheroidal-celled cancer, primary
<i>Tongue</i>						
Case 14	Axillary glands, tuberculous	67			—	—
" 15	{ Axillary glands, tuberculous	49			—	—
" 16	{ Breast, tuberculous	31			—	—
<i>Uterus</i>						
Case 16	Mucous membrane	42			—	Squamous-celled cancer
" 17	Muscular wall	51, 61			—	—
" 18	" "	51			—	—
<i>Placenta</i>						
Case 18	Placental tissue	37, 42			—	—
<i>Ovary</i>						
Case 19	Ovarian cyst	40, 51			—	—
<i>Prostate</i>						
Case 20	Prostatic tissue	15, 40			—	—
<i>Thyroid</i>						
Case 21	Thyroid tissue	28, 40			—	—
<i>Stomach</i>						
Case 22	Mucous membrane	47, 50			—	—
<i>Large intestine</i>						
Case 23	Mucous membrane	29, 32			—	—
<i>Voluntary muscle</i>						
Case 24	Muscle	38			—	—
" 25	" "	34, 45			—	—

Pairs of figures indicate duplicate analyses.

(32 mg.). The remaining tumours of the breast were all spheroidal-celled cancers; satisfactory material for comparison, which could be regarded as normal mammary glandular tissue, was not obtained from any of these specimens. In Case 9 the tumour was very cellular in parts; the lymphoid tissue of the axillary glands was almost wholly replaced by carcinoma, with some fibrous interspaces, and these glands gave the highest figure (154 mg.) of the whole series. Case 10 showed cellular islands, with much fibrous tissue; the glutathione content was low (32 mg.). The primary tumour in Case 11 was a scirrhous, with much fibrous tissue, and the glands showed in parts considerable fibrosis; their glutathione content (68 mg.) was lower than in Case 9. In Case 12 the primary tumour showed a considerable amount of fibrous tissue; the invaded glands showed large areas of carcinoma cells, and the low result (38 mg.) is difficult to explain. Case 13 is included for the sake of comparison; it was a tuberculous breast, with involvement of some of the axillary glands. This case was thought to be one of cancer until the sections were examined.

(3) *Tongue*. Both tumours (Cases 14 and 15) were typical epitheliomas, showing very little necrosis. The material taken for comparison, named "mucous membrane" in the table, was the epithelium dissected off with a varying amount of the underlying fibrous tissue.

(4) *Uterus*. The normal muscular wall from which the mucous membrane was removed (Cases 16 and 17), and the non-malignant tumour (fibromyoma, Case 16) developed from it, are very closely similar in structure and show the same glutathione content.

(5) *Ovary*. The tumour was a papilliferous ovarian cyst of the usual type from which the mucoid contents were removed as far as possible; the non-cellular material consists of the fibrous trabeculae upon which the epithelium rests.

(6) *Placenta*. This was a full-time specimen<sup>1</sup>; a considerable part of the material consists of the connective tissue of the villi. The glutathione content is rather low (37 to 42 mg.).

(7) *Prostate*. This adenoma showed fibrous and glandular portions, and possibly the unequal distribution of these accounts for the large difference between the two analyses (15 and 40 mg.).

(8) *Thyroid*. Here also the analyses differ more than is usual. The gland showed distended vesicles, and no papilliferous growth; the very large portion of the sections occupied by colloid suggests that, if the colloid contains no glutathione, the amount of this compound in the epithelium of the vesicles must be very large.

(9) *Stomach* and (10) *Large intestine*. These samples of mucous membrane were dissected off through the submucous coat, as in the specimens from the rectum, and showed similar proportions of cellular and non-cellular material. The former specimen was taken from the healthy portion of stomach wall

<sup>1</sup> We are indebted for this to Dr Holland, of St Luke's Hospital, Chelsea. The analysis was begun 45 minutes after the expulsion of the placenta.

resected in a case of gastric ulcer, the latter from a piece of intestine removed on account of prolapse from a colostomy opening.

(11) *Voluntary muscle*. This was the pectoral muscle removed in amputation of the breast.

The general impression that one obtains from examining sections of almost all these tissues, and from considering at the same time the quantitative chemical results, is that on the whole the amount of glutathione varies with the amount of cellular, or perhaps rather nuclear, material present, and is quite independent of the normal or neoplastic nature of the tissue.

#### DISCUSSION.

The data in Table I certainly give no indication of any deficiency of glutathione in tumours. Thus in the series of comparative analyses of the mucous membrane, and cancers, of the rectum, the amounts in the former range from 35 to 56 mg., and in the latter from 52 to 59 mg. % in spite of the difficulty, alluded to above, of obtaining this cancer tissue without a large quantity of non-cellular material. The value of the figures for cancers of the breast is lessened by the impossibility of obtaining normal glandular tissue for comparison; but the high figures (154 and 68 mg.) given by axillary glands in which the lymphoid tissue is replaced wholly or largely by cancer cells shows that these must contain a considerable amount of glutathione. Again, the two epitheliomas of the tongue (Cases 14 and 15), which offered particularly compact masses of carcinoma, gave high figures (70 to 81 mg.). Of the innocent tumours, the uterine fibroid (Case 16) gave the same result as the muscular wall of the uterus, with which it is almost identical in structure. But the conclusion to be drawn from this investigation is illustrated most clearly by the fact that no less than 14 out of the 20 largest amounts of glutathione were found in cancers.

#### *Fixed —SH groups.*

The solid residue after extraction of the soluble glutathione was in every case tested for insoluble —SH groupings. The intensity of the colour produced bore no constant relation, either direct or inverse, to the amount of glutathione extracted. The residue of the specimen of breast-tissue giving an extractable glutathione content of 0.9 mg. % gave a negative result with this test. When the soluble glutathione values are arranged in order of magnitude the residues of the tissues to which the seven highest figures correspond gave strong reactions for fixed —SH without exception. However, two of the tissues having a glutathione value of 51 mg. % gave a weak and a very strong reaction respectively. Nor if the tissues are grouped according to a histological classification is the agreement between type and residual nitroprusside reaction at all close.

*Starch as indicator.*

Glutathione values as determined by the starch end-point method gave, in the case of human tissues, figures of the order of 40 to 50 % greater than those obtained with the nitroprusside technique (Table II). The prostatic adenoma, however, yielded starch results which were in the one case twice as great and in another nearly four times greater than the nitro-prusside figures. The nature of the substances which combine with iodine after the nitro-prusside end-point is reached has not been determined.

Table II. *Human tissues.*

Comparison of glutathione content (mg. per 100 g. fresh tissue) as determined by nitroprusside and by starch.

N=nitroprusside as indicator. S=starch as indicator.

Case	N	S	Case	N	S	Case	N	S
1	48	60	7	85	130	18	63	75
	35	38	8	32	59	17	51	71
	59	73	9	60	102	18	42	63
	56	72		154	231		37	50
2	56	72	10	32	60	19	51	82
	47	67	11	29	49		40	77
	52	77		68	104	20	40	80
3	50	77	12	25	34		15	55
	55	82		38	55	21	28	43
	51	74	13	31	58		40	52
	55	70		67	109	22	47	68
4	50	69		49	78		50	63
	51	66	14	77	84	23	29	41
	41	55		81	89		32	42
5	52	80		42	52	24	38	48
	45	71	15	70	90	25	45	53
	51	71	16	51	63		34	39
6	91	113		61	69			
	0.9	2.7		50	62			

*Concentration of glutathione in human tissues.*

The figures in Table I show that the glutathione content of the human tissues examined is rather low (generally from 25 to 60 mg. %) in comparison with the organs analysed by previous workers [Tunncliffe, 1925; Voegtlin and Thompson, 1926; Holmes, 1926; and Yaoi and Nakahara, 1926], which are chiefly the liver, spleen and kidney of the rabbit, rat and fowl. These latter organs contain generally from 150 to 300 mg %. Certainly one might expect that the organs of animals smaller than man, in which the metabolism per unit body-weight is greater, would show a higher concentration of glutathione. These considerations show the importance of using the corresponding *human* normal tissues as controls on analyses of human tumours.

## TISSUES OF THE FOWL, AND THE ROUS SARCOMA.

Forty estimations in all were carried out, and Rous tumours from 27 fowls were analysed. The liver, pancreas, kidney, lung, and voluntary muscle were taken as control tissues. The results are shown in Table III.

Table III. *Tissues of the fowl, and the Rous sarcoma.*

N = nitroprusside as indicator. S = starch as indicator.  
mg. glutathione in 100 g. fresh tissue.

Fowl	Inoculation site	Rous tumour		Liver		Pancreas		Muscle		Kidney		Lung	
		N	S	N	S	N	S	N	S	N	S	N	S
1	Intramuscular	0.0	20	85	106	32	80	13	16	65	83	—	—
"	"	3.3*	9.6*	—	—	—	—	—	—	—	—	—	—
2	"	12	14	151	194	21	48	16	18	98	114	—	—
"	"	16*	24*	—	—	—	—	—	—	—	—	—	—
3	"	34	54	136	154	36	59	24	26	97	97	—	—
4	"	24	27	175	195	61	95	15	21	—	—	—	—
5	Subcutaneous	3.1	—	128	143	53	81	17	19	130	158	—	—
6	"	0.0	21	—	—	—	—	—	—	—	—	—	—
7, 8, 9	"	13	18	—	—	53	86	—	—	—	—	39	51
10	"	34	47	—	—	—	—	—	—	—	—	—	—
11	"	19	29	—	—	—	—	—	—	—	—	—	—
12, 13	"	17	24	—	—	46	87	—	—	—	—	—	—
14	"	17	30	—	—	—	—	—	—	—	—	—	—
15	"	14	21	—	—	—	—	—	—	—	—	—	—
16, 17, 18	"	28	41	—	—	—	—	—	—	—	—	—	—
19, 20, 21, 25, 26	"	28	52	—	—	—	—	—	—	—	—	—	—
22, 23, 24, 27	"	36	51	—	—	40	76	—	—	—	—	—	—

\* These tumours to the naked eye appeared especially necrotic.

*Technique.* In 17 estimations on the Rous tumours, sometimes pooled, of 27 fowls, the amounts of glutathione found ranged from nil to 36 mg. %. The first six samples of tumour analysed were produced by inoculation in the manner which has for some reason become conventional in dealing with the Rous tumour, namely, by injection deeply into the pectoral muscle. The material obtained thus is a mixture of living and necrotic tumour, mucoid ground-substance, and disintegrated muscle, and from this it is impossible to isolate the healthy tumour tissue required for analysis. The amounts of glutathione are very irregular (nil to 34 mg.), and it is impossible to take from such material pieces for microscopical examination which are fair samples of the whole. It was found very much better to inoculate the tumour in the way almost always employed for rat and mouse tumours, that is, subcutaneously. The tumour after inoculation under the skin of the breast spreads as a sheet of fairly compact cellular tissue over the pectoral muscle, which it does not at first invade. The sheet soon invades the skin, but is easily scraped off it, and in sections is found to contain no muscle, very little necrotic material, and a comparatively small proportion of ground substance. The tumour is in a suitable state for examination 6 days after inoculation in fowls weighing 300–400 g., and in 11 days in older birds (1000–1500 g.). The amounts of glutathione found in the tumours grown subcutaneously range from 3 to 36 mg. %; the single case (No. 6) in which none was found was a bird which died during the night and was examined on the following day. The tumour grows freely in White Leghorns, which were used in most of these experiments. The birds were bled under anaesthesia before the removal of the various tissues.

## DISCUSSION.

Of the five control tissues, the liver gives by far the highest results (up to 175 mg.). The wide range of variations in these tissues (liver, 85 to 175 mg.; pancreas, 21 to 61 mg.; kidney, 65 to 130 mg.) is similar to that found in the organs of rats by Voegtlin and Thompson (*e.g.* kidney, 19 to 156 mg.; liver, 135 to 261 mg.). Table III shows clearly that there is nothing whatever peculiar about the glutathione content of the Rous tumour. Of the 17 samples of it analysed, 11 gave a figure higher than the lowest amount found in voluntary muscle (13 mg.), and no less than 6 contained more than the least amount in the pancreas (21 mg.), which latter is certainly a very cellular organ. Yaoi and Nakahara [1926] have maintained recently that the Rous tumour "is very characteristic in showing no, or exceedingly feeble response to the nitroprusside test. The tumour is in this way readily separable from other malignant tumours as well as from normal tissue," and they have suggested that this absence or great deficiency of glutathione is associated with the resistance of the tumour to drying and to the action of glycerol. The results given in Table III suggest that the Japanese workers have been misled by using the glutinous and largely non-cellular material which can be obtained in abundance when a Rous tumour inoculated into muscle reaches an advanced stage of growth. They make no mention of any microscopical control of their chemical work. From Tables I and III one sees that a Rous tumour may contain as much glutathione as is present in the pancreas of the fowl bearing it (fowl 3) or in some normal human tissues (rectal mucous membrane, voluntary muscle, placenta).

Incidentally, the results show that glutathione estimations have no bearing on the question whether the Rous tumour is or is not similar in nature to mammalian tumours.

*Fixed —SH groups.* After extraction of the Rous tumour the residue was tested for —SH groups. With two exceptions (the tumours from fowl 4 and the pooled tumours from fowls 16, 17 and 18) a distinct to strong colour was observed. In the two exceptions only a weak colour resulted. The extracts from these two tumour specimens gave a moderately large yield of glutathione, namely, 24 and 28 mg. %. Here again as in the case of the human tissues there was found no constant correspondence between extracted glutathione and residual —SH groupings. The tumour with a glutathione value of only 3.1 left a residue which gave practically no colour with the reagents. In many of these tests the colour obtained was of an extraordinarily fugitive nature.

*Starch as indicator.* In most of the analyses the difference between the results given by nitroprusside and by starch is similar to that found in the case of human tissues. But two instances are peculiarly instructive. In fowls 1 and 6, titration with nitroprusside gave nil, and with starch 20 or 21 mg. % "glutathione" (Table III). Yaoi and Nakahara, who used starch alone as indicator in all their quantitative estimations, found 20 mg. % as a maximum

in the Rous tumour. But as their qualitative nitroprusside tests were almost or quite negative, it is clear that their quantitative results with starch are quite unreliable as estimations of glutathione.

*Glutathione and anaerobiosis.*

It has been stated that a deficiency or absence of glutathione in tumours is in accordance with Warburg's observations on anaerobiosis in tumours, and corroborates his teaching. But, apart from the fact that such deficiency of glutathione does not occur, one may point out that it is not required at all by Warburg's theory of malignancy. He has never claimed that tumour cells do not oxidise fats or amino-acids; and in a recent paper [Warburg, Wind and Negelein, 1927] has calculated that not more than two-thirds of the carbohydrate used by a tumour is dealt with anaerobically. Hence in a tumour the metabolism of fats, of proteins, and of one-third of the carbohydrates, might be carried out by those same factors, including glutathione, which are active in normal tissues.

SUMMARY.

Analyses of normal tissues and of tumours in man, and in the fowl, show that tumours are not distinguished by any deficiency in glutathione.

We are much indebted to Dr H. J. B. Fry, Pathologist to the Cancer Hospital, in whose laboratory the sections of human tumours were prepared and examined.

One of us, I. H., has received a grant from the British Empire Cancer Campaign, during the carrying out of this work.

REFERENCES.

- Holmes (1926). *Biochem. J.* **20**, 812.  
Tunnicliffe (1925). *Biochem. J.* **19**, 194.  
Voegtlin and Thompson (1926). *J. Biol. Chem.* **70**, 793, 801.  
Warburg, Wind and Negelein (1927). *J. Gen. Physiol.* **8**, 519.  
Yaoi and Nakahara (1926). *Gann. (Japan. J. Cancer Res.)*, **20**, 51.



## CI. OBSERVATIONS ON THE RENAL THRESHOLD FOR GLUCOSE.

By ROBERT LINDSAY MACKAY.

*From the Wolverhampton and Staffordshire General Hospital.*

*(Received May 4th, 1927.)*

IN the course of a recent investigation on the blood-glucose changes in general anaesthesia, certain observations were made which have a direct bearing on the question of the so-called renal threshold for glucose.

The renal threshold may be defined as being that level or concentration of sugar in the blood which has to be reached or exceeded before any easily recognisable quantity of sugar will appear in the urine. The idea of a renal threshold for a substance presupposes that until a certain concentration shall have been reached in the blood none will appear in the urine.

The prevailing opinions on this subject may be briefly summarised as follows: Macleod [1926] considers the renal threshold for sugar to lie between 0.160 % and 0.180 %; Todd [1923] from 0.170 % to 0.180 %; MacLean [1925] from 0.160 % to 0.170 %; de Wesselow [1925] regards the normal as 0.180 %, but he recognises considerable variations above and below this figure. Host [1925], in an interesting study on carbohydrate tolerance in pregnancy, has shown that, while early in pregnancy the renal threshold is normal, later on the threshold falls and permits glycosuria to occur independently of hyperglycaemia. Goto and Kuno [1921] placed the upper level of the renal threshold at 0.170 %, but they were unable to state the lower level. Benedict, Osterberg and Neuwirth [1918] deny the significance of a renal threshold, stating that their results (on two normal men) indicate that the threshold doctrine should be abandoned. Most of the foregoing workers have based their conclusions generally on studies of carbohydrate tolerance, or on the giving of standard doses of glucose; in other words, their studies are based on the behaviour of exogenous glucose.

Certain difficulties stand in the way of a correct estimation of the renal threshold: (a) even when blood and urine are removed as nearly at the same time as possible they cannot be considered as simultaneous specimens, (b) while a blood-sugar curve is falling the relationship between the sugar concentration in the blood and in the urine may be quite different from what it is while the curve is ascending, (c) the rate of urine formation is an unknown and variable but important factor, (d) it is difficult (unless blood is removed very often, e.g. at 2-minute intervals) to make quite certain about the highest point of a curve, e.g. after a glucose meal, or during or after anaesthesia.

## METHOD OF INVESTIGATION.

It was decided to approach this subject from a different view-point, and to study the behaviour of endogenous, as opposed to exogenous, sugar in the blood. For some time it has been generally known that a marked hyperglycaemia may be caused by the administration of a general anaesthetic, such as chloroform or ether, the hyperglycaemia being presumably a manifestation of the glycogenolytic action of the anaesthetic which causes a "mobilisation" in the sugar depôts of the body. The source of the hyperglycaemia is thus endogenous, unlike that caused by a glucose meal.

The method of investigation chosen was as follows. Forty-four successive subjects, for operation under general anaesthesia by ether or chloroform-ether mixture, had their blood-sugar estimated at intervals of 15–20 minutes, before, during and after anaesthesia. The cases were not selected in any way, and none of them was known to suffer from diabetes or from renal trouble. The variety of the lesions for which operation was performed was characteristic of that found on the surgical side of any large general hospital. In the case of each patient a blood-sugar curve was drawn up, and the curve in Fig. 1

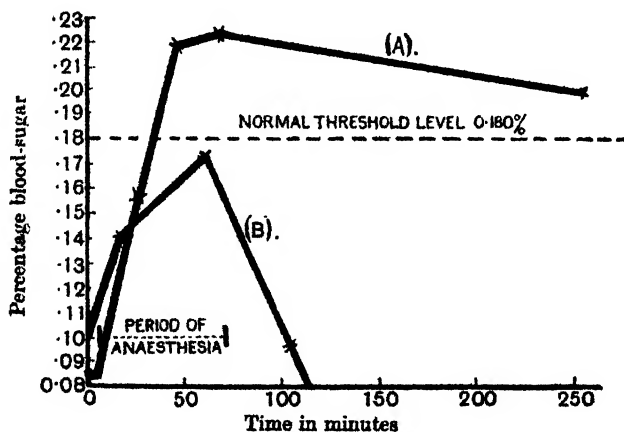


Fig. 1.

(A) Blood-sugar curve of case No. 3.

A. W., *art.* 35 years. Hydatids in liver.

Open ether anaesthesia for 70 minutes.

Sugar absent from urine both before and after operation.

This curve, which is quite typical of the blood-sugar alterations in anaesthesia, may be compared on the same scale with

(B) Blood-sugar curve after 50 g. glucose meal as carried out by MacLean [1925].

(case 3) is characteristic of the curves in the 44 cases under consideration. On this graph there is also shown, for comparison, the curve of the blood-sugar after a 50 g. glucose test meal in normal individuals as carried out by MacLean [1925]. All the essential details, such as nature of the anaesthetic chosen (mainly ether), period of administration, nature of disease and of operation,

age of patient, etc., were noted and tabulated. In addition, the first urine passed after recovery from the anaesthetic was collected and examined for sugar by (1) the fermentation test, and (2) Bertrand's modification of Fehling's test. The pre-operative urine was also examined for sugar and in each case was found to be negative. The initial and maximum blood-sugar percentages were noted, and, in addition, an approximate estimate was made of the length of time during which the blood-sugar might reasonably be considered to be at a hyperglycaemic level. This level was fixed arbitrarily at 0.180 %.

The essential results are shown in Table I.

Table I. *To show increase in blood-sugar due to anaesthesia, the period in minutes during which the blood-sugar was above 0.180 %, and the presence or absence of post-operative glycosuria.*

Number in series	% B.S. before opera- tion	Maxi- mum % B.S.	Minutes above 0.180 %	Sugar in urine	Number in series	% B.S. before opera- tion	Maxi- mum % B.S.	Minutes above 0.180 %	Sugar in urine
1	0.177	0.297	100	+	23	0.109	0.187	5	+
2	0.122	0.241	10	+	24	0.143	0.253	200	+
3	0.083	0.222	220	-	25	0.160	0.181	.	+
4	0.115	0.196	.	+	26	0.095	0.187	15	+
5	0.146	0.386	250	-	27	0.090	0.184	.	-
6	0.160	0.216	45	+	28	0.100	0.211	35	-
7	0.146	0.181	.	+	29	0.106	0.156	.	+
8	0.137	0.206	60	-	30	0.100	0.287	50	+
9	0.184	0.227	30 +	+	31	0.050	0.143	.	+
10	0.100	0.216	5	+	32	0.118	0.156	.	+
11	0.150	0.184	5	+	33	0.075	0.181	.	-
12	0.120	0.181	.	-	34	0.118	0.175	.	+
13	0.093	0.243	140	-	35	0.098	0.133	.	+
14	0.118	0.156	.	+	36	0.165	0.206	25	-
15	0.175	0.320	200	+	37	0.110	0.175	.	+
16	0.141	0.156	.	+	38	0.143	0.193	15	-
17	0.162	0.266	50	-	39	0.118	0.137	.	+
18	0.088	0.214	10	-	40	0.112	0.150	.	+
19	0.099	0.137	.	+	41	0.106	0.237	80	+
20	0.109	0.225	200	+	42	0.037	0.193	5	+
21	0.109	0.227	45	+	43	0.110	0.175	.	-
22	0.106	0.147	.	-	44	0.191	0.291	180	+

#### DISCUSSION.

It is necessary now to examine this table in detail. Of the 44 cases 30 showed glycosuria and 14 did not.

(A) Of the 30 glycosuric cases, in 11 the blood-sugar never reached 0.180 %, i.e. in 37 % of cases sugar was excreted by the kidney at a level below the normal threshold value. Of the 14 cases where no sugar was excreted, in 12 cases (i.e. in 86 %) the blood-sugar had risen above the normal threshold of 0.180 %. Thus 37 % in the first case and 86 % in the second do not seem to accord with the doctrine of the renal threshold. It may be objected, however, that in a number of cases the blood-sugar may have been above 0.180 % for such a short period that practically no sugar would be excreted in that time. To overcome this legitimate objection a further analysis has been made.

(B) Taking those cases where the blood-sugar as shown on the curves

lay above 0.180 % for over 20 minutes one finds that of 17 such cases in 7 (*i.e.* 41 %) there was no glycosuria—still too high a percentage to permit the wholehearted acceptance of this theory. Further, of the 13 cases where the blood-sugar was continuously under 0.180 %, 11 were found to excrete sugar, *i.e.* 87 %.

Therefore, when an arbitrary average figure of 0.180 % is taken as the renal threshold for sugar, and when the results are considered with relation to that figure, one fails to get any weighty evidence in favour of the theory and finds much to gainsay its validity.

(C) If, instead of taking the renal threshold as 0.180 %—a fixed level—one considers only those cases outside the average range of renal thresholds as given by the workers quoted earlier—say 0.160 % to 0.190 %—then the findings are as follows. Of 30 cases of glycosuria, in 14 the blood-sugar was above 0.190 % and in 7 it was below 0.160 %, *i.e.* 14 are in conformity with the theory and 7 are against. Of the 14 sugar-negative cases 9 had a blood-sugar above 0.190 %, and 4 below 0.160 %. This gives 4 cases in consonance with the theory compared with 9 against it.

In other words, no matter how the foregoing table is analysed, whether the renal threshold is taken as a fixed level of 0.180 %, or as a fixed range of from 0.160 % to 0.190 %, and whether the time factor is included or omitted, no positive substantial evidence can be given to support the theory. Rather may it be said that the weight of the evidence is all against it. Case No. 5, for example, had a blood-sugar curve which rose from 0.146 % to 0.386 %, received an anaesthetic for 85 minutes and then a rectal saline with 5 % glucose, and had a blood-sugar concentration over 0.200 % for over 4 hours, and yet the first urine passed when consciousness was regained neither reduced Fehling's solution nor fermented with yeast.

The deduction made therefore from this analysis is that the results in half of the cases examined do not accord with the doctrine of a renal threshold for sugar of about 0.180 %. When, in addition, it is recollected that traces of sugar or related carbohydrates can be demonstrated in normal urine, a further doubt is thrown upon the validity of the theory, so far as sugar is concerned. The nature of the evidence here adduced, while not consistent with the present idea of a sugar threshold, is not sufficiently strong to enable one to submit another theory as attractive, but it demonstrates the need for some re-statement. For the moment, then, it is suggested that the present position should be summarised thus. The renal threshold for sugar, if there is one, is a much more variable factor than is generally supposed. Under the influence of an anaesthetic, it varies greatly between one normal person and another. It even appears to change during the course of a pregnancy [Host, 1925]. For all the evidence to the contrary, it may even vary frequently to slight degrees in the normal person at different times and in different circumstances. No physiological fluids in the body have an absolutely constant composition, but appear to be influenced, if only slightly, by various physical and metabolic

processes and influences in the attempt to maintain a condition of equilibrium. The renal threshold for sugar is not unlikely to be one such varying factor.

#### SUMMARY.

An investigation is described carried out with the object of determining the relationship between post-anaesthetic glycosuria and the blood-sugar changes due to the anaesthetic. The explanation of the results obtained in terms of the doctrine of the renal threshold for sugar at about 0.180 % proving unsatisfactory, it is suggested that this theory requires re-statement, if not, indeed, abandonment.

This investigation, which was suggested to me by Prof. E. P. Cathcart, F.R.S., was carried out in the biochemical laboratory of the Wolverhampton and Staffordshire General Hospital by kind permission of Dr S. C. Dyke, Pathologist to the Hospital, for whose friendly advice and criticism I am much indebted. Mr Deanesly, B.Sc., F.R.C.S., and Mr Cholmeley, F.R.C.S., Honorary Surgeons to the Hospital, kindly accorded me every facility in their theatres and wards, while all members of the anaesthetics staff gave me their cordial co-operation.

#### REFERENCES.

- Benedict, Osterberg and Neuwirth (1918). *J. Biol. Chem.* **34**, 209, 217.  
Goto and Kuno (1921). *Arch. Int. Med.* **27**, 224.  
Host (1925). *Lancet*, i, 1022.  
MacLean (1925). Diagnosis and treatment of glycosuria and diabetes.  
Macleod (1926). Carbohydrate metabolism and insulin (London), p. 210.  
Todd (1923). Clinical diagnosis by laboratory methods (Saunders Co., London), p. 366.  
de Wesselow (1925). Chemistry of the blood in clinical medicine, p. 88.





**I. A. R. I. 76.**

IMPERIAL AGRICULTURAL RESEARCH  
INSTITUTE LIBRARY  
NEW DELHI.

[illegible]